XXVIII. THE ENZYMES OF WASHED ZYMIN AND DRIED YEAST (LEBEDEFF). III. PEROXYDASE, CATALASE, INVERTASE AND MALTASE.

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Peroxydase.

On the assumption that hydrogen peroxide and peroxydase might possibly be capable of oxidising some degradation product of sugar, Bach [1906] submitted sugar to the simultaneous action of hydrogen peroxide, peroxydase and an enzyme capable of decomposing sugar. He chose for his experiments the fermenting enzyme of yeast and employed zymin. Being however of the opinion that yeast contains no peroxydase he made use in his investigations of a peroxydase prepared from horse-radish. Although unable to observe any of the oxidation processes the probability of which he had anticipated, he found that when the peroxydase used was previously boiled the amount of carbon dioxide evolved was invariably more than when it was not boiled. This led him to conclude that the presence of active peroxydase had a deterrent effect on the fermenting power of zymin.

The authors find that fresh English brewery yeast contains active peroxydase. When however the yeast is dried for 17 hours at a temperature of 37° it no longer gives the reaction. On washing the dried yeast however the presence of peroxydase can again be detected.

Dried Munich yeast (Schroder) behaves like dried English yeast and does not react for peroxydase when unwashed, but regains that activity on being washed. Whether the peroxydase in yeast has a deterrent effect on the fermentation the authors are at present not prepared to say. It is to be observed that Bach in his experiments used a peroxydase from an outside source (horse-radish) and the presence of an agent in that preparation that would have a retarding effect on fermentation and which could be destroyed by boiling is not impossible.

Experimental.

Experiment 1. Ten g. of pressed English brewery yeast were washed four times to remove adhering wort, etc., and suspended in 300 cc. of distilled water. The washing in this and all the other experiments was done by stirring up the yeast in pots with 120 cc. of distilled water for the first time and 100 cc. for all subsequent times and centrifuging. Into two small Erlenmeyer flasks were introduced:

Flask 1. 10 cc. of yeast suspension (equivalent to 0·1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 (10 per cent. dilution of 3 per cent. H_2O_2 neutralised to litmus).

Flask 2. 10 cc. of yeast suspension + 1 cc. 1 per cent. p-phenylenediamine.

Flask No. 1 showed the first sign of a colouration in one minute and assumed an intense violet colouration in three minutes.

Flask No. 2 remained unchanged.

It is evident from this experiment that English yeast contains an active peroxydase.

Experiment 2. A sample of the yeast used in the previous experiment was then dried for 17 hours at a temperature of 37° and one gram of the dried yeast suspended in 100 cc. of distilled water. Into two flasks were introduced:

Flask No. 1. 10 cc. of yeast suspension (0·1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 (10 per cent. dilution of 3 per cent. H_2O_2).

Flask No. 2. 10 cc. of yeast suspension + 1 cc. 1 per cent. p-phenylenediamine.

Although flask No. 1 after one minute assumed a slightly darker colour than the control flask No. 2, it did not show any violet colouration and the reaction was decidedly negative. The repetition of the above experiments gave similar results and we may conclude that drying inhibits the activity of the peroxydase in the yeast.

Experiment 3. Some dried English yeast was washed four times; this process removes soluble phosphates, the coenzyme of zymase and, doubtless, other substances. Into two flasks were introduced:

Flask No. 1. 10 cc. of washed yeast suspension (0·1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. dilute H_2O_2.

Flask No. 2. 10 cc. of unwashed dried English yeast suspension (0·1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. dilute H_2O_2.

Flask No. 1 showed a colouration after 2 minutes. Flask No. 2 showed no colouration after 5 minutes. This experiment shows that the activity of the dried yeast can be restored by washing.

Experiment 4. 5 g. of dried English yeast were washed four times. The first washings amounted to 32 cc. Six Erlenmeyer flasks were taken and in each were placed 10 cc. of washed yeast suspension (0·1 g. of dried yeast) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. dilute H_2O_2.

To flask No. 1 nothing was added.
" No. 2 0·6 cc. of the washings was added.
" No. 3 1 cc. " " "
" No. 4 1·5 cc. " " were added.
" No. 5 5 cc. " " "
" No. 6 5 cc. of the neutralised washings were added.

No. 1 showed a colouration after one minute, No. 2, No. 3 and No. 4 after 3, 4 and 4 minutes respectively. No. 5 and No. 6 showed no colouration after 15 minutes. This experiment shows that the addition of the washings inhibits the action of the peroxydase in yeast.

Experiments similar to Nos. 3 and 4 were carried out with dried Munich yeast (Schroder) and zymin, and the results obtained were identical with those obtained with English dried yeast. It is however to be observed that various samples of zymin gave reactions of different intensity.

It is evident from these results that after the yeast cell has been dried or treated with acetone washing removes some substance which has an inhibiting effect on the peroxydase of the yeast. The washings not only inhibit the action of the peroxydase in yeast but also exert a deterrent effect on that of milk as shown by Experiment 5.

Experiment 5. Into two flasks were introduced:

Flask No. 1. 1 cc. dilute H_2O_2 + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. of milk + 5 cc. of water.

Flask No. 2. 1 cc. dilute H_2O_2 + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. of milk + 3 cc. of water + 2 cc. of washings from dried Munich yeast.

Flask No. 1 showed a colouration immediately. Flask No. 2 showed no colouration after 5 minutes.

It was further observed that the action of the inhibiting agent seems to increase in a higher proportion than that of the peroxydase as the amount of yeast is increased.

The following experiment illustrates this point.

Experiment 6. Flask No. 1. 0·05 g. of dried English yeast suspended in 10 cc. of H_2O + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2.

Flask No. 2. 0·2 g. of dried English yeast suspended in 10 cc. of water + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2.

Flask No. 1 showed the first signs of a colouration only after 4 minutes and eventually assumed a violet colouration which, although not so intense as in the case of washed yeast, was well marked. Flask No. 2 showed no colouration after 15 minutes.

Several other substances have been found, the addition of which to washed dried Munich yeast (Schroder) inhibits the action of the peroxydase. These are beef broth (0·1 cc.), sodium lactate (0·5 cc. of 1 per cent.), peptone (1 cc. of 1 per cent.), alkalis and acids. Beef broth previously treated with H_2O_2 for 20 minutes still has an inhibiting effect. As to the acids and alkalis, small traces of either will inhibit the action of the peroxydase in yeast as shown by the following experiment.

Experiment 7. Flask No. 1. 10 cc. of washed dried Munich yeast (Schroder) suspension (0·1 g.) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 0·5 cc. N/10 KOH.

Flask No. 2. 10 cc. of washed dried suspension (0·1 g.) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 0·5 cc. N/10 H_2SO_4.

Flask No. 3 contained 10 cc. of washed dried yeast (Schroder) suspension (0·1 g.) + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2.

No. 3 showed a colouration after two minutes. No. 1 and No. 2 showed no colouration after 5 minutes.

As the washings of dried yeast are slightly acid to litmus the possibility that the acidity of the washings acted as the inhibiting agent on the peroxydase suggested itself, but it was experimentally proved that this was not the case.

Experiment 8. In this experiment the washings were carefully neutralised to litmus.

Flask No. 1. 10 cc. of washed dried Munich yeast suspension + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 3 cc. H_2O.

Flask No. 2. 10 cc. of washed dried Munich yeast suspension + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 1·5 cc. of neutralised washings + 1·5 cc. H_2O.

Flask No. 3. 10 cc. of washed dried Munich yeast suspension + 1 cc. 1 per cent. p-phenylenediamine + 1 cc. H_2O_2 + 3 cc. neutralised washings.

No. 1 showed a colouration after 2 minutes while No. 2 and No. 3 showed no colouration after 5 minutes.

Precisely the same result was obtained when the washings were made neutral to phenolphthalein.

Washed and unwashed Munich dried yeast, washed and unwashed English dried yeast, washed and unwashed fresh English brewery yeast give no reaction with p-phenylenediamine and with benzidine in absence of H_2O_2, which proves the absence of an oxydase. Similarly the above-mentioned yeasts under the same conditions give no reaction for tyrosinase.

Catalase.

In the experiments of Bach mentioned earlier in this paper this experimenter also observed that the amount of oxygen evolved by the catalase of the zymin was almost equal to the theoretical amount capable of being liberated from the hydrogen peroxide. In order to ascertain with precision whether the washing of dried Munich yeast influenced the catalase, experiments were carried out as follows: 0·1 g. of dried Munich yeast (Schroder) was introduced into a small Erlenmeyer flask connected with an azotometer filled with mercury over which the liberated oxygen could be collected and measured. 25 cc. of hydrogen peroxide were run in from a burette passing through the stopper of the flask, and the volume of the oxygen liberated and the time taken for the liberation of the gas noted. Then a second experiment was carried out in which 25 cc. of the hydrogen peroxide were treated in the same apparatus with excess of acidified $KMnO_4$, and the volume of oxygen liberated again measured. The available oxygen of the hydrogen peroxide, i.e. half the amount evolved on treatment with $KMnO_4$, was thus ascertained and compared with the volume evolved by the action of the catalase of the yeast. The washed dried Munich yeast was treated in a similar way.

Experiment 1. 0·1 g. of unwashed dried Munich yeast liberated from 25 cc. of H_2O_2 17·1 cc. of oxygen in 5 minutes.

Acidified $KMnO_4$ and 25 cc. of the same H_2O_2 liberated 33·6 cc. of oxygen.

Experiment 2. 0·1 g. of washed dried Munich yeast liberated from 25 cc. of another sample of H_2O_2 11 cc. of oxygen in 5 minutes.

Acidified $KMnO_4$ and 25 cc. of the same H_2O_2 gave 21·9 cc. of oxygen.

It is evident from these results that the catalase in the dried Munich yeast is capable of liberating the entire available oxygen of the H_2O_2 and that the washing of the yeast makes no difference either to the volume of the oxygen liberated or to the rate of evolution.

Invertase and Maltase.

Experiments were next made to ascertain whether by repeated washing at ordinary room temperature it was possible to remove all traces of invertase and maltase from dried Munich yeast (Schroder) and zymin. The investigations were carried out by means of two different sets of experiments. In one set the yeast was washed six times. The first washings were boiled in order to destroy any invertase and maltase they might contain and added to the washed yeast, the mixture being then digested with cane sugar and maltose respectively for 17 hours at 25° and the CO_2 evolved, if any, noted. One flask containing glucose and another containing water in addition to the mixture of washed yeast and boiled washings were also digested at the same time for comparison. In the event of the invertase and maltase being entirely washed out no fermentation of cane sugar or maltose could be expected to take place, whereas if hydrolysis occurred the resulting hexoses would be fermented by the zymase in presence of the coenzyme added in the washings.

In the second set of experiments the cane sugar and the maltose were respectively digested with the washed yeast without the addition of the washings for 3 hours at a temperature of 25°. The suspensions were then filtered and the filtrate examined in order to ascertain whether any hydrolysis had occurred. In this case, no coenzyme being present, no fermentation could take place.

1. Invertase.

Experiment 1. The Munich dried yeast used in this experiment was washed six times.

No. 1. 25 cc. (5 g.) of washed Munich yeast + 20 cc. (1 g.) of cane sugar solution + 30 cc. of boiled washings + 0·5 cc. of toluene.

No. 2. 25 cc. (5 g.) of washed Munich yeast + 20 cc. (1 g.) of glucose + 30 cc. of boiled washings + 0·5 cc. of toluene.

No. 3. 25 cc. (5 g.) of washed Munich yeast + 20 cc. H_2O + 30 cc. boiled washings + 0·5 cc. toluene.

The digestion was continued for 17 hours at 25°.

The contents of all three flasks fermented and the following volumes of CO_2 were evolved:

Time	Flask No. 1	Flask No. 2	Flask No. 3
After 30 minutes	8·9 cc.	8·2 cc.	3·6 cc.
,, 60 ,,	14·8	14·1	5·5
,, 90 ,,	18·8	17·7	7·2
,, 17 hours	54·8	57·3	16·0

An experiment in which zymin was substituted for the dried Munich yeast washed six times was also carried out and the results obtained were similar. In 17 hours 25·3 cc. of CO_2 were evolved from the cane sugar, 25·2 cc. from the glucose, and 4·9 cc. from the autofermentation of the zymin.

These results show that the rate of evolution and the volume of the CO_2 produced from cane sugar are almost the same as from glucose, and it may therefore be concluded that the amount of invertase left in the dried Munich yeast and the zymin after washing is very appreciable.

The above results are confirmed by the following set of experiments.

Experiment 2. The dried Munich yeast used was washed six times.

Flask No. 1. 25 cc. (5 g.) of washed dried Munich yeast + 20 cc. (1 g.) of cane sugar solution + 0·5 cc. of toluene.

Flask No. 2. 25 cc. (5 g.) of washed dried Munich yeast + 20 cc. of water + 0·5 cc. of toluene. The contents of the two flasks were incubated for 3 hours at 25° and were then filtered, and the ratio

$$\frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}}$$

of filtrate No. 1 was determined, the rotation of filtrate No. 2 being reckoned for in calculating the actual ratio.

$$\text{Ratio } \frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}} \text{ of filtrate No. 1} = \frac{-0{\cdot}373}{0{\cdot}500} = -0{\cdot}746.$$

$$\text{Ratio } \frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}} \text{ of invert sugar} = -0{\cdot}800.$$

Experiments similar to Experiment 2 were carried out with dried Munich yeast washed with a mixture of water and toluene and with zymin washed both with water only and with water and toluene. The following are the results obtained:

With zymin washed with water $\quad \text{Ratio} = \frac{-0{\cdot}586}{0{\cdot}736} = -0{\cdot}796.$

" " " and toluene $\quad \text{Ratio} = \frac{-0{\cdot}629}{0{\cdot}760} = -0{\cdot}827.$

With dried Munich yeast washed with toluene and water

$$\text{Ratio} = \frac{-0{\cdot}614}{0{\cdot}730} = -0{\cdot}840.$$

All the above results show that the entire cane sugar was inverted during the three hours.

In view of these results it became of interest to ascertain whether there was any difference in the rate of inversion of cane sugar between washed and unwashed zymin. Zymin was chosen because small quantities of that preparation in a concentrated solution of sugar do not commence fermenting for

some time and consequently it is possible to ascertain the amount of sugar inverted during that time and compare it with the amount inverted in the same time by an equal weight of zymin after being washed.

Experiment 3. The mixtures were incubated for 20 minutes at a temperature of 25°.

Flask No. 1. 0·1 g. of unwashed zymin suspended in 20 cc. of water + 50 cc. of 10 per cent. cane sugar solution.

Flask No. 2. 0·1 g. of washed zymin suspended in 20 cc. of water + 50 cc. of 10 per cent. cane sugar solution.

No fermentation was observed during the twenty minutes. The flasks were then placed in boiling water for twenty minutes in order to stop any further action of the invertase. After cooling to the room temperature they were made up to 100 cc. and filtered and the reducing powers of the filtrates calculated in terms of glucose were determined.

Reduction of filtrate No. 1 = 1·04 g. of glucose per 100 cc.
" " No. 2 = 0·67 g. " " "

It is thus seen that about $\frac{1}{5}$ of the total cane sugar was inverted by the unwashed zymin during the twenty minutes and that 64·4 per cent. of the amount inverted by the unwashed zymin was inverted by the washed preparation. This would suggest that the amount of invertase left behind even after six washings is a very considerable fraction (approximately 65 per cent.) of that originally present.

2. Maltase.

A series of experiments similar to those carried out with cane sugar were made with maltose.

Washed dried Munich yeast (Schroder) and washed zymin readily fermented maltose on addition of the boiled washings. The volumes of CO_2 were as follows:

	Maltose	Glucose	Autofermentation
Munich dried yeast	58·0 cc.	57·3 cc.	16·0 cc.
Zymin	25·1	25·2	4·9

As from cane sugar the rate of evolution and volume of the CO_2 evolved from maltose are almost equal to those given by glucose, and consequently the amount of maltase left behind in the preparations after washing must also be very considerable.

The resulting ratios $\frac{\text{Rotation in 400 mm. tube}}{\text{Reduction (g. glucose per 100 cc.)}}$ of filtrates obtained after digesting maltose without the addition of the washings for 3 hours at 25° with dried Munich yeast (Schroder) and zymin respectively, each of these preparations washed with water alone and with a mixture of water and toluene, were:

Dried Munich yeast washed with water only $\frac{+1{\cdot}095}{0{\cdot}550} = +1{\cdot}99.$

" " " " and toluene $\frac{+1{\cdot}848}{0{\cdot}680} = +2{\cdot}7.$

Zymin washed with water only $\frac{+2{\cdot}360}{0{\cdot}574} = +4{\cdot}1.$

" " " " and toluene $\frac{+2{\cdot}886}{0{\cdot}490} = +5{\cdot}89.$

Ratio for glucose + 2·1. Ratio for maltose + 9·8.

It appears from these figures that the whole of the maltose was converted into glucose by the dried Munich yeast while only a part of the maltose was converted by the zymin. Washing with a mixture of water and toluene seems however to have made no great difference to either preparation.

The idea of the possibility of removing the maltase from the zymin by a more thorough process of washing suggested itself and an experiment was accordingly instituted in which the zymin was washed 8 times. Each time it was vigorously shaken up in the pot for several minutes and the last time it was placed in a shaker for an hour previous to being centrifuged. On addition of the boiled washings the mixture fermented maltose freely at a rate almost equal to that produced with glucose. It is therefore evident that the most thorough method of washing will not entirely remove the maltase from zymin at ordinary room temperature.

As in Experiment 3 with cane sugar, 50 cc. of 10 per cent. maltose were also digested with 0·1 g. of washed and unwashed zymin respectively for 20 minutes. The resulting filtrates obtained showed the following reductions per 100 cc. calculated in terms of glucose:

Washed zymin 2·2 per cent. Unwashed zymin 2·2 per cent.

In this case although only a part of the maltose was converted into glucose the amounts hydrolysed were equal in both cases, and therefore the quantity of maltase removed by the washing can only have been very small.

SUMMARY.

1. Fresh English yeast reacts with H_2O_2 and p-phenylenediamine for peroxydase.

2. Dried yeast (both English and Munich) does not react for peroxydase with p-phenylenediamine and H_2O_2 but on being washed it regains that activity.

3. The addition of the washings and some other reagents to washed dried yeast inhibits the action of peroxydase.

4. Washing does not affect the activity of the catalase of dried yeast (Schroder).

5. The power of hydrolysing cane sugar is partially but not entirely removed from zymin and dried Munich yeast (Schroder) by washing at ordinary room temperature, whilst the power of hydrolysing maltose is not affected.

REFERENCE.

Bach, A. (1906), *Ber.* **39**, 1664.

XXIX. AN INVESTIGATION INTO THE PHYSICO-CHEMICAL MECHANISM OF HAEMOLYSIS BY SPECIFIC HAEMOLYSINS. No. II. THE ELECTRICAL CONDUCTIVITY OF SENSITISED CORPUSCLES AND THE ACTION OF INORGANIC FERMENTS OR METAL-SOLS UPON THEM.

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In my previous communication [1913] I pointed out that in the process of haemolysis by specific haemolysins, the amboceptor molecules are at first adsorbed into the pores in the walls of the erythrocytes and subsequently a true chemical combination takes place between these and the molecules of the corpuscular walls.

Observations have since been carried on to determine the electrical conductivity of the sensitised corpuscles as compared with the unsensitised ones. The following methods were adopted.

Sheep's corpuscles after being washed with normal saline were treated with six times the working dose of anti-sheep amboceptor and the mixture kept for a variable period, from two to twenty-four hours, in the ice-chamber. Subsequently the corpuscles were centrifuged and thoroughly washed with normal saline. In a second series of observations the sensitised as well as the unsensitised corpuscles were suspended in 5 % glucose solution and then separated by centrifugalisation.

The conductivity of the corpuscles was measured by Kohlrausch's method and the following results were obtained. In all cases the conductivity was calculated at 26° C.

TABLE I.

The electrical conductivity of the sediment of corpuscles from the emulsion in normal saline.

(*a*)	(1)	Unsensitised corpuscles	K (26° C.) $\times 10^5 = 360$
	(2)	Sensitised corpuscles	K (26° C.) $\times 10^5 = 300$
(*b*)	(1)	Unsensitised corpuscles	K (26° C.) $\times 10^5 = 300$
	(2)	Sensitised corpuscles	K (26° C.) $\times 10^5 = 120$
(*c*)	(1)	Unsensitised corpuscles	K (26° C.) $\times 10^5 = 190$
	(2)	Sensitised corpuscles	K (26° C.) $\times 10^5 = 160$

TABLE II.

The electrical conductivity of the sediment of corpuscles from the emulsion in 5 % glucose solution in distilled water.

(*a*)	(1)	Unsensitised corpuscles	K (26° C.) $\times 10^5 = 250$
	(2)	Sensitised corpuscles	K (26° C.) $\times 10^5 = 100$
(*b*)	(1)	Unsensitised corpuscles	K (26° C.) $\times 10^5 = 70$
	(2)	Sensitised corpuscles	K (26° C.) $\times 10^5 = 38$

It will be seen from the above tables that the adsorption of amboceptor by erythrocytes was followed by a diminution of the electrical conductivity of the corpuscles and this was specially marked when the sediment of corpuscles was obtained from an emulsion in isotonic glucose solution.

In the case of the sediment from the suspension in normal saline, the conductivity is mainly due to the ions still in contact with the corpuscular walls. Therefore the corpuscular walls of the sensitised corpuscles obstruct the passage of the ions more than the unsensitised ones. In the case of the sediment from glucose solution, the conductivity is mostly due to the envelopes. From these the conclusion follows that the walls of the sensitised corpuscles conduct electricity worse than those of the unsensitised ones.

From the above it may be assumed that the diminution of conductivity of the corpuscular walls in the case of sensitised corpuscles is due to the action of amboceptor on the walls and therefore that at least a portion of the amboceptor is in some sort of combination with the corpuscular walls in sensitised corpuscles.

Action of inorganic ferments or metal-sols upon sensitised corpuscles.

A series of experiments was undertaken to determine the action of catalysts, such as animal charcoal and platinum black, upon sensitised corpuscles. No haemolysis was brought about by their action. Colloidal solution of iodine (Collo-Iode of Dubois) also gave negative results. Various

metal-sols were mixed in varying concentration with the sensitised corpuscles. The following have, up to the present time, been used. Electargal and electro-selenium prepared by Clin and Co. (Paris), collo-sol silver and collo-sol mercury prepared by Oppenheimer and Sons.

Up to now, all such experiments with colloids have failed to bring about haemolysis of the sensitised corpuscles.

Conclusions.

(1) In the process of adsorption of amboceptor by the erythrocytes, it is in the corpuscular walls that the molecules of the amboceptor remain adsorbed.

(2) The electrical conductivity of the corpuscular walls of the amboceptor-loaded corpuscles is less than that of normal erythrocytes.

(3) Metal-sols, as well as other catalysts, such as animal charcoal, platinum black, colloidal iodine do not bring about any haemolysis of sensitised corpuscles.

I am deeply indebted to Col. Sutherland, I.M.S., and Dr G. C. Mitter of the Medical College, Calcutta, for providing me with the haemolytic antisera for my experiments, and to Professor Nilratan Dhar of the Presidency College, Calcutta, for assisting me in determining the electrical conductivities.

REFERENCE.

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XXX. ON THE ESTIMATION OF β-HYDROXYBUTYRIC ACID.

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The experiments described in this paper were undertaken in order to test those methods for the estimation of β-hydroxybutyric acid which are applicable to mixtures of its two optical isomers. Two such methods are available, dependent upon the conversion of the acid into crotonic acid, and into acetone, respectively.

The production of crotonic acid has been applied as a method of estimation by Darmstädter [1903], Ryffel [1905], and Pribram [1912]. Darmstädter's method has been shown by Shindo [1907] to be very inaccurate. Pribram obtained 99% of the theoretical amount of crotonic acid in as little as 350 cc. of distillate. In the present investigation a large number of estimations were made by Pribram's method, and the yield was not more than 96% in from 700 to 1200 cc. of distillate. The form of the curve obtained is very similar to that given below for the formation of acetone from β-hydroxybutyric acid; in the later stages of the distillation the crotonic acid comes over more and more slowly. In Ryffel's method this difficulty was avoided by calculating the ultimate result of the distillation from the rate of evolution observed in fractions of the first 400 cc. of distillate.

The conversion of β-hydroxybutyric acid into acetone by oxidation with sulphuric acid and potassium bichromate was applied as a method of estimation by Shaffer [1908]; the acetone produced was estimated by means of iodine and thiosulphate in the ordinary way. It seems preferable to employ the method of Scott-Wilson [1911] for determining the amount of acetone, since the reagent introduced by him is less likely than is iodine to be affected by the presence of other substances. This combination of the methods of Shaffer and Scott-Wilson has been used in the investigation of a series of cases of acidosis [Kennaway, Pembrey, and Poulton, 1913].

Results obtained with the Scott-Wilson method.

The acetone is precipitated by a reagent containing mercuric cyanide, silver nitrate, and caustic soda; the amount of mercury in the precipitate is estimated by titration with potassium sulphocyanide. On the assumption that one molecule of acetone combines with five atoms of mercury, Scott-Wilson obtained results which were about 3 % too low. The following experiments were carried out with acetone from the bisulphite compound (Kahlbaum).

TABLE I.

		Mg. acetone in 10 cc.
I.	(1) 0·0990 gm. acetone weighed and diluted to 1 litre ...	0·990
	(2) 100 cc. of above solution estimated by iodine method ...	(1) 0·981
		(2) 0·981
	(3) 10 cc. estimated by Scott-Wilson method. Mean of ten estimations (max. 0·980, min. 0·960)	0·9718
	(4) 5 cc. estimated by Scott-Wilson method. Mean of ten estimations (max. 0·986, min. 0·963)...	0·9707
II.	(1) 0·0747 gm. acetone weighed and diluted to 1 litre ...	0·747
	(2) 100 cc. of above solution estimated by iodine method ...	(1) 0·722
		(2) 0·722
	(3) 10 cc. of above solution estimated by Scott-Wilson method ...	(1) 0·719
		(2) 0·713
III.	Acetone solution of unknown strength:	
	(1) 100 cc. estimated by iodine method	0·8180
	(2) 10 cc. estimated by Scott-Wilson method	0·8177

In Scott-Wilson's experiments, the precipitation of acetone by the mercuric cyanide reagent was found to be complete in 10 minutes if the reagent were added in the proportion of not less than 25 cc. for 1 mg. of acetone. These estimations were made with a $\frac{N}{1000}$ acetone solution (i.e. 0·58 mg. in 10 cc.). Since the concentration of acetone in distillates from urine is often much lower than this, the question of the relation between this concentration and the rate of precipitation requires some further testing.

TABLE II.

Precipitation of acetone in different concentrations with the same time of precipitation.

Portions of 10 cc. of the acetone solution given under (II) above were precipitated with Scott-Wilson reagent, the concentration being in some cases lowered by addition of water, as shown in the third column below. The precipitation was in all cases allowed to proceed overnight.

Acetone solution, cc.	Scott-Wilson reagent, cc.	Total volume, cc.	Result of estimation	
			Cc. KCNS used	Mg. acetone
10	25	35	12·40	0·719
,,	25	35	12·30	0·713
,,	25	35	12·20	0·707
,,	20	30	12·35	0·716
,,	20	30	12·30	0·713
,,	20	100	12·20	0·707
,,	20	100	12·05	0·699
,,	20	200	12·10	0·702
,,	20	200	11·90	0·690
,,	20	300	11·40	0·661
,,	20	300	10·90	0·632
,,	20	300	10·70	0·620

These results show that the precipitation can be rendered incomplete and irregular by sufficient dilution. The rate of precipitation was tested as follows:

TABLE III.

Precipitation of acetone at the same concentration with different times of precipitation.

Mixtures of Acetone solution ... 10 cc.
Scott-Wilson reagent ... 20 ,,
Water ... ... 70 ,,

Hours allowed for precipitation	Result of estimation	
	Cc. KCNS used	Mg. acetone
½	12·00 12·00	0·696
1	12·30 11·95	0·703
1½	11·90 11·90	0·690
2	12·00	0·696
2½	12·00	0·696
5	12·10 12·20	0·7047
18	12·30 12·20 12·05	0·7064

The differences observed in this series are almost within experimental error. A full investigation of the relations between different concentrations of acetone and of mercuric cyanide, and the rate of precipitation, would obviously be very laborious. In practice, this question does not give much trouble. With a little experience, it is easy to judge from the rate at which the precipitate develops whether an excessive amount of acetone has been added to the amount of reagent taken. In any case of doubt, one can (1) add more reagent to one of two duplicates; (2) keep one duplicate for a longer time before filtration; or (3) keep the filtrate to see whether any further precipitate develops in it. It is well to obtain distillates which contain more than 0·7 mg. acetone in 100 cc. (see Table II), to aim at using 30 cc. reagent for 1 mg. acetone, and to leave the mixtures when possible overnight before filtration.

Except where it is otherwise stated, all the acetone estimations given in this paper were made by the Scott-Wilson method.

Results obtained with Shaffer's method.

(*a*) *The estimation of β-hydroxybutyric acid.*

Shaffer [1908] obtained approximately correct yields (98·8 to 103·3 %), using a preparation of β-hydroxybutyric acid the purity of which was not tested in a satisfactory manner. Cooke and Gorslin [1911] showed that they could obtain concordant duplicates by this method, but did not attempt to establish its accuracy. Mondschein [1912] obtained an average yield of 97·8 %, but he gives no particulars of the preparation of the acid used in his experiments.

The preparation used in the estimations given below was made from the impure inactive acid (Kahlbaum). The liquid was diluted with water, filtered, and extracted with ether; the extract was distilled in vacuo, the residue neutralised with caustic soda, and the salt recrystallised five times from alcohol. It gave the following result on combustion[1]:

0·1600 g.; 0·0806 g. H_2O; 0·2240 g. CO_2.

	C %	H %
Found	38·20	5·60
Calculated for $C_4H_7O_3Na$	38·09	5·55

When weighed amounts of this salt were treated by Shaffer's method,

[1] I am indebted to Mr V. Steele for carrying out this analysis.

the yield of acetone obtained was from 90 to 96·5 % of the theoretical amount. The concentration of acetone in successive portions of distillate becomes less and less until the amount, if any be present, is too small for estimation. (Table IV and Fig. 1.)

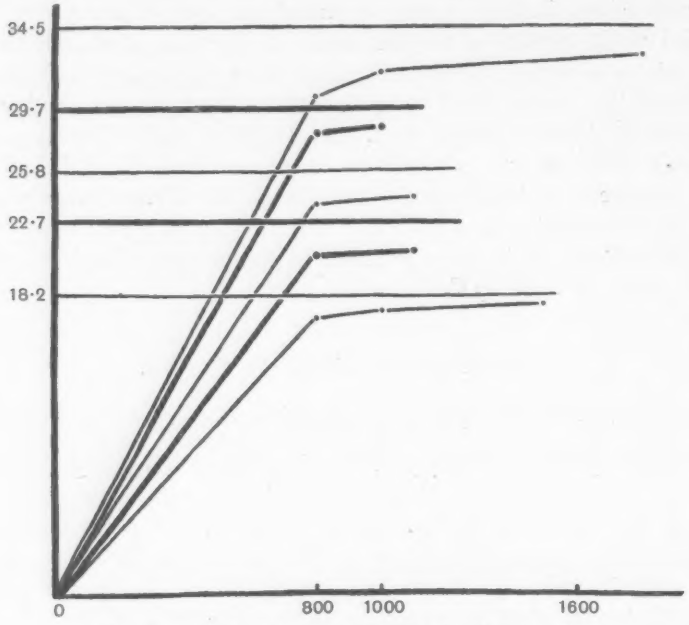

Fig. 1. Formation of acetone from β-hydroxybutyric acid.

Abscissae represent cc. distillate.
Ordinates represent mg. acetone.
Horizontal lines show the theoretical amounts of acetone.

Various attempts were made to increase the yield by altering the manner in which the oxidation was carried out. If the concentration of sulphuric acid be increased to six volumes per cent., which is twice that recommended by Shaffer, the reaction comes to an end sooner, but the total yield is not increased (see Expts. VII and VIII, Table IV). Slow addition of the bichromate produces a somewhat higher result than the more rapid addition of the same amount (Fig. 2). Theoretically, 1 part of acetone should be produced by 1·7 parts of bichromate. Large excess of bichromate certainly diminishes the yield, as Shaffer stated; on the other hand, the theoretical

amount of bichromate produces only about $\frac{2}{3}$ of the theoretical yield of acetone (see Expt. VI, Table IV; and Fig. 2). Between these two extremes of excess and defect, there is a wide range over which the amount of bichromate seems to have no regular influence on the result. This is shown in Table V, in which the yields are arranged in descending order.

TABLE IV.

Sodium β-hydroxybutyrate distilled with 3 volumes % sulphuric acid, and potassium bichromate.

Expt.	Distillate, cc.	Acetone found, mg.	Acetone calculated, mg.	Yield %	Potassium bichromate, mg. used	Potassium bichromate, mg. calculated
I	500	18·94				
	300	1·63				
	300	0·26				
	1100	20·83	22·69	91·9	250	40
II	800	81·78				
	500	trace				
	1300	81·78	91·2	90	700	155
III	800	28·10				
	200	0·17				
	1000	28·27	29·64	95·4	600	50
IV	800	29·69				
	200	1·95				
	800	1·22				
	1800	32·86	34·47	95·3	440	59
V	800	23·74				
	300	0·49				
	1100	24·23	25·77	94	400	44
VI	800	39·45			100	
[Effect of theoretical amount of bichromate]	800	16·54			150	
	300	0·72				
	1900	56·71	60·39	93·9	250	103
VII	800	26·216				
[6 vols. % sulphuric acid]	800	Nil				
	1600	26·216	27·93	93·9	300	48
VIII	800	12·934				
[6 vols. % sulphuric acid]	800	Nil				
	1600	12·934	13·96	92·6	200	24

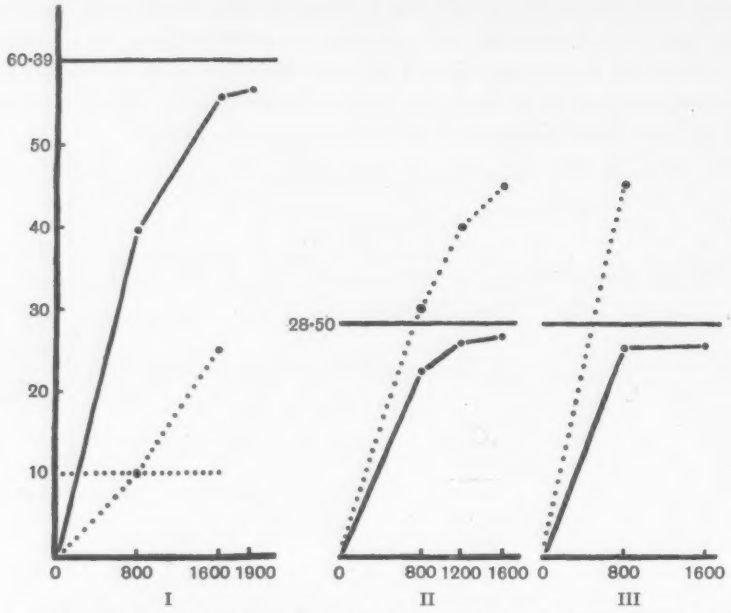

Fig. 2. Effect of amount of bichromate on the yield of acetone from β-hydroxybutyric acid.
Abscissae represent cc. distillate.
Ordinates represent mg. acetone or cg. bichromate.
Continuous lines represent acetone; the horizontal lines show the theoretical yield.
Dotted lines represent bichromate.
Curve I. Effect of theoretical amount of bichromate (horizontal dotted line).
Curves II and III. Duplicate determinations on urine with added sodium β-hydroxybutyrate. Slow (II) and rapid (III) addition of bichromate.

TABLE V.

Effect of amount of bichromate upon the yield of acetone from sodium β-hydroxybutyrate.

Quotient $\frac{\text{Bichromate used}}{\text{Bichromate calculated}}$	Yield %
12	95·4
7	95·3
9	94·0
2·5	93·9
6	93·9
8	92·6
6·5	92·1
6·25	91·9
4·6	90·0
Mean 6·6	93·2

When the sodium salt is added to urine, and the urine then treated in the ordinary way by Shaffer's method, the yield is as a rule somewhat higher than that given by a pure solution (Table VI); probably the increase is due to concentration of the fluid brought about by the bulky precipitate of lead compounds[1].

TABLE VI.

Sodium β-hydroxybutyrate added to urine and treated by Shaffer's method.

Expt.	Distillate, cc.	Acetone found, mg.	Acetone calculated, mg.	Yield %
I	800	23·90		
	300	0·08		
	1100	23·98	25·69	93·4
II	800	28·72		
	300	0·44		
	1100	29·16	31·65	92·1
III	800	16·07		
	400	1·30		
	400	0·17		
	1600	17·54	18·52	94·6
IV	800	16·80		
	800	0·30		
	1600	17·10	18·19	94
V	800	16·88		
	200	0·44		
	500	0·23		
	1500	17·55	18·19	96·5
VI	800	15·72		
	700	1·71		
	600	trace		
	2100	17·43	18·19	95·8
VII	800	16·72		
	600	0·62		
	1400	17·34	18·19	95·3

It is then easy to show that Shaffer's method does not give a quantitative yield of acetone, but it is of course much more difficult to find out whether the acetone obtained from urine by oxidation is derived wholly from β-hydroxybutyric acid, and there seems to be no satisfactory way of doing this. However, the following experiment was made to investigate this question. A diabetic urine containing 3·4% sugar, 0·228% aceto-acetic acid, and

[1] In these and all other cases where substances were added to urine, the amount yielded by the urine alone was of course subtracted from the result.

0·925 % β-hydroxybutyric acid, was acidified and extracted with ether until the extract was optically inactive. The second distillate from the residue when treated by Shaffer's method gave with Scott-Wilson's reagent a barely perceptible trace of precipitate, such as might well have been due to incomplete extraction of the β-hydroxybutyric acid. This experiment of course shows merely that the ether-insoluble substances did not yield acetone.

After nearly all the estimations given in this paper had been made[1], Shaffer and Marriot [1913] published some very similar results on the formation of acetone from β-hydroxybutyric acid. They obtained from 90 to 94 % of the theoretical amount; by very slow oxidation this was increased to from 95 to 97 %.

(*b*) *The estimation of aceto-acetic acid.*

The accuracy of Shaffer's method as a means of estimating aceto-acetic acid, which is greatly preponderant over acetone in urine [Arnold, 1900], appears never to have been tested. The estimations made with this substance were not altogether satisfactory, as difficulty was experienced in establishing a constant boiling point for the ester employed. However, the following experiments were carried out.

3·2525 gram aceto-acetic ester was saponified in the cold with the calculated amount of N caustic soda (25 cc.), and diluted to 1 litre (Solution I).

	Acetone mg.		
	Found by Scott-Wilson estimation	Calculated from weight of ester	Yield %
10 cc. of above solution distilled by Shaffer's method	14·15	14·50	97·6
25 cc. " " " " "	35·96	36·25	99·0

This solution gave low results when added to urine. Probably some ester was still present, which was decomposed to acetic acid by the strong ammonia used in Shaffer's method. The solution was therefore shaken out five times with ether to remove ester; it had then taken up acetone from the ether, but the results obtained before and after addition to urine were compared. (Solution II.)

Solution II. 25 cc. distilled gave 54·37 mg. acetone.
25 cc. added to urine gave 53·12 mg. acetone = 97·7 %.

Another preparation (III) gave the following results:

50 cc. distilled gave 10·526 mg. acetone.
50 cc. added to urine gave 10·522 mg. acetone.

[1] A preliminary communication was given to the Physiological Society, Nov. 15th, 1913. [Kennaway, 1913.]

The recovery of acetone from urine was tested as follows:

10 cc. acetone solution precipitated with Scott-Wilson's reagent gave 0·597 mg.
100 cc. added to urine gave ... ... 6·037 mg. = 101 %.

Analyses of mixtures of sodium aceto-acetate and sodium β-hydroxybutyrate.

	Acetone, mg.		Yield
	Calculated	Found	%
1. Aceto-acetic acid (I)	36·25	35·23	97·2
β-hydroxybutyric acid	31·94	29·41	92·1
2. Aceto-acetic acid (I)	36·25	35·23	97·2
β-hydroxybutyric acid	25·76	24·23	94·0
3. Added to urine			
Aceto-acetic acid (III)	6·737	6·826	101·0
β-hydroxybutyric acid	28·50	26·34	92·1

A combination of the Shaffer and Scott-Wilson methods.

This may be illustrated by stating in detail the quantities used in a particular estimation. The method is taken from the papers of Shaffer [1908], Cooke and Gorslin [1911], and Scott-Wilson [1911], with various modifications which are mentioned below.

25 cc. of diabetic urine giving a moderately strong ferric chloride reaction was placed in a 250 cc. flask, diluted with water, and mixed with 50 cc. basic lead acetate solution[1] and 10 cc. strong ammonia. A drop of dilute sulphuric acid in the supernatant fluid showed that excess of lead was present. The mixture was made up to 250 cc. 100 cc. of the filtrate was made faintly acid with 9 cc. 20 vol. % sulphuric acid, and made up to 250 cc. 100 cc. of this solution was placed in the distilling flask with 400 cc. water, 15 cc. concentrated sulphuric acid and talc, and 300 cc. distilled off at constant volume. The distillate was made up to 500 cc., and two portions of 50 cc. precipitated with 20 cc. Scott-Wilson reagent.

800 cc. was then distilled off at constant volume with addition of 400 cc. 0·2 % potassium bichromate and water; the distillate was made up to 1 litre, and two portions of 50 cc. precipitated with 20 cc. Scott-Wilson reagent.

300 cc. was then distilled off at constant volume with addition of water,

[1] The U.S.P. solution used by Shaffer is prepared as follows: 180 g. lead acetate is dissolved in 700 cc. water; the solution is boiled for half an hour, while 110 g. of litharge is added in small portions. The solution is made up to 1 litre. The amount required is in most cases from one-half to twice the volume of urine.

and made up to 500 cc.; 100 cc. of this with 15 cc. reagent gave no precipitate on standing overnight.

The Scott-Wilson titrations gave the following results:

(1) Aceto-acetic acid + acetone.

$$\text{KCNS}\ \left.\begin{matrix}9{\cdot}45\text{ cc.}\\9{\cdot}45\text{ cc.}\end{matrix}\right\} = 0{\cdot}548\text{ mg. acetone in } \tfrac{1}{100}\times\tfrac{10}{11}\times\tfrac{10}{11}\times 25 = 0{\cdot}4\text{ cc. urine.}$$

(2) β-hydroxybutyric acid.

$$\text{KCNS}\ \left.\begin{matrix}10{\cdot}70\text{ cc.}\\10{\cdot}75\text{ cc.}\end{matrix}\right\} = 0{\cdot}622\text{ mg. acetone in } \tfrac{1}{100}\times\tfrac{10}{11}\times\tfrac{10}{11}\times 25 = 0{\cdot}2\text{ cc. urine.}$$

The estimation of such small quantities does not involve any considerable error. If the last two titration figures given above had been 10·5 cc. and 10·9 cc., and the day's volume of urine had been 7000 cc., the total amount of β-hydroxybutyric acid reckoned separately from the two titrations would have been 38·3 or 39·8 grams. The titrations as a rule do not differ by more than 0·2 cc.

The reasons for taking as much as 25 cc. of urine when the ultimate estimation is carried out upon the equivalent of 0·2 or 0·4 cc. are (1) the errors of the series of measurements are lessened by working with large volumes; (2) it is important that the concentration of acetone in the distillate should not be too low (see Table II)[1]; (3) with some urines, but not with all, the conversion of a larger quantity of β-hydroxybutyric acid into acetone takes place more rapidly and completely than that of a smaller quantity (Fig. 3).

It is most convenient to have from 0·5 to 1 mg. of acetone in 25 to 50 cc. of distillate. 1 mg. of acetone requires 17·3 cc. N/100 KCNS. The amount of acetone bodies in a urine can be judged roughly by colour tests. If Rothera's reaction gives a strong colour within a few seconds, probably more than 0·25 % aceto-acetic acid is present; the reaction is still given by a concentration of 0·0005 % or less [Hurtley, 1913]. The ferric chloride reaction is not obvious unless more than 0·07 % aceto-acetic acid is present. In order to form some idea of the amount of β-hydroxybutyric acid, one may remember that in well-marked cases of acetonuria this acid forms from 60 to 80 % of the total acetone bodies [Neubauer, 1910]; if this percentage were 66, the concentrations of acetone in the two distillates obtained by the method given above would be equal.

The urine is measured into the flask first, and diluted before adding the basic lead acetate; there is then less frothing. The froth can be

[1] Direct distillation into the reagent, as practised by Scott-Wilson with normal urine, would not be practicable in pathological cases.

dispersed, when brought up into the neck of the flask, by rubbing with a glass rod and piece of filter-paper[1].

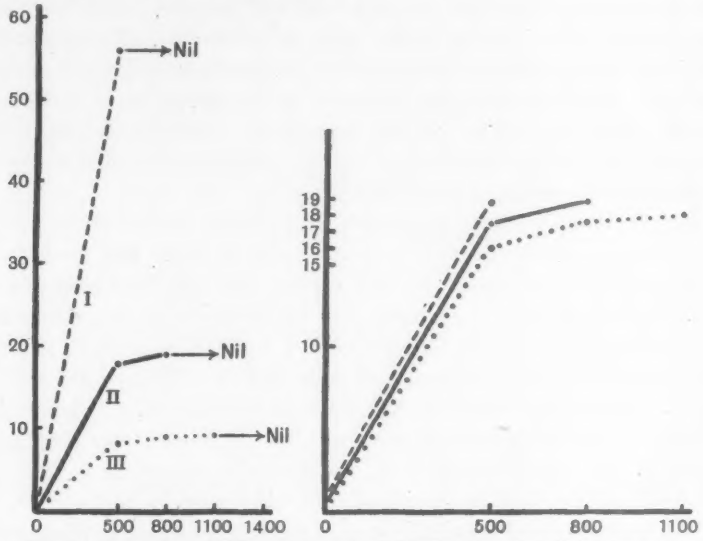

Fig. 3. Conversion of different amounts of β-hydroxybutyric acid into acetone.
Abscissae represent cc. distillate.
Ordinates represent mg. acetone.
I. Estimation on equivalent of 12 cc. urine.
II. ,, ,, ,, 4 ,,
III. ,, ,, ,, 2 ,,

In the right-hand figure, the results given in curves I and III are calculated for 4 cc. urine, to facilitate comparison.

Estimation on equivalent of—	12 cc. urine	12 cc.	4 cc.	2 cc.
1st distillation, 500 cc.	55·98	55·97	17·57	8·062
2nd ,, 300 ,,	Nil	Nil	1·14	0·823
3rd ,, 300 ,,			Nil	0·14
4th ,, 300 ,,				Nil
Mg. acetone, total	55·98	55·97	18·71	9·025
	÷3=18·66			×2=18·05

The apparatus which has been used in these estimations is shown in Fig. 4. The distillate from the first flask is passed through a second flask, as in Scott-Wilson's method, containing about 30 cc. 40 % caustic soda and an equal volume of water, with a little talc; this is kept at more or less constant volume by a small flame, and towards the close of the distillation

[1] A drop of ether is of course highly effectual, but must on no account be used, as ordinary ether, and even that sold as "distilled over sodium," contains quite enough acetone to affect a Scott-Wilson estimation.

is boiled down to a small volume. This flask must rest on a sand-bath, as it is apt to crack on wire gauze. The distillate is received into a 500 cc. or 1000 cc. measuring flask; the condenser and delivery-tube should be in one piece, as no rubber junction at this point is satisfactory. The measuring flask is connected with a water trap in a 50 cc. cylinder; it is well to let the water suck back here soon after the start of the distillation, to make sure that the apparatus is tight. In carrying out the oxidation, it is generally convenient to keep the funnel filled with 0·2 % bichromate, and if necessary to add water or a few cc. of 5 % bichromate.

In the estimation of β-hydroxybutyric acid, a second distillation is necessary to show whether the formation of acetone is completed. The result is in many cases nil, or an amount too small to estimate; in other cases it may be as much as 10 % of the whole. The obvious method of avoiding this lengthy distillation and the large volume of distillate is to carry out the oxidation with a reflux condenser and bulbs, and then distil off the acetone formed. The results obtained thus were always too low, this being due probably to loss of uncondensed acetone when changing to the distillation apparatus. In estimations on urine, the formation of acetone is as a rule completed more rapidly than when the pure acid is used.

The second distillation with hydrogen peroxide described by Shaffer was not found to make any considerable difference in those cases where it was tested (Table VII). When a somewhat lower result is obtained from the

TABLE VII.

Comparison of results with Iodine and Scott-Wilson methods.

Milligrams acetone.

	From aceto-acetic acid + acetone		From β-hydroxybutyric acid			
			Before H_2O_2		After H_2O_2	
	Iodine method	Scott-Wilson method	Iodine method	Scott-Wilson method	Iodine method	Scott-Wilson method
Urine 1	15·46	15·48	41·82	41·90	42·30	41·83
,, 2	16·19	15·98	39·40	38·64	38·68	38·78
,, 3	14·75	14·93	52·22	51·11	50·76	50·60
,, 4	15·59	15·83	50·76	50·60	49·05	49·45
,, 5	5·560	5·568	17·88	17·57	17·52	17·40
,, 6	42·51	41·76	111·19	106·15		
,, 7	6·16	6·32	21·75	21·97		
,, 8	9·91	9·12	49·31	46·40		
,, 9	4·83	5·18	24·65	24·48		
Blood 1	11·12	11·34	24·17	20·88		
,, 2	12·33	12·29	28·04	24·52		

second distillation, the diminution may be due to loss of acetone rather than to removal of other substances; possibly these are eliminated by the method of distillation through caustic soda adopted in these experiments (Fig. 4). When blood is treated in the same way as urine, besides acetone, other substances which combine with iodine are undoubtedly formed (Table VII).

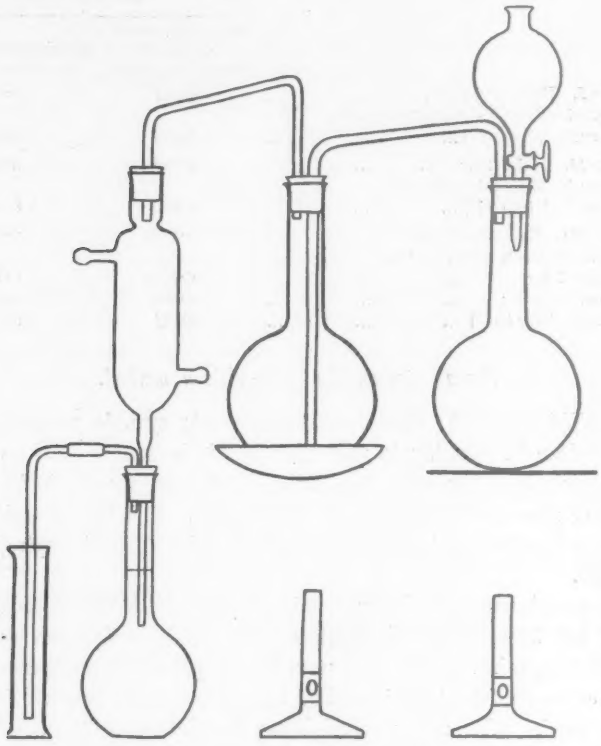

Fig. 4.

Scott-Wilson showed that urine when decomposed by bacterial action yielded more acetone, or some substance reacting like acetone with his reagent. This change occurs slowly even when the urine is preserved with thymol in a cold room, and remains acid and apparently fresh (Table VIII); the β-hydroxybutyric acid is as a rule unaffected. Thymol when oxidised with bichromate and sulphuric acid yields some substance which is precipitated by Scott-Wilson's reagent, but comparative experiments show that the amounts

of thymol present in the filtered and diluted urine are not sufficient to affect the result. Chloroform cannot be used, as it causes reduction of the reagent, and the results are too high.

TABLE VIII.

	In 100 cc. urine	
	Aceto-acetic acid + acetone as acetone, grams	β-hydroxybutyric acid as acetone, grams
Urine I. Estimated fresh.	0·187	0·812
Kept with thymol at room temperature		
6 months later (acid)	0·229	0·819
Urine II. Estimated fresh.	0·062	0·1911
Kept with thymol in cold room		
5 months later (acid)	0·068	0·1907
Urine III. Estimated fresh.	0·1305	0·300
Kept with thymol in cold room		
10 days later	0·1298	0·2994
10 weeks later	0·138	0·311
12 weeks later (acid)	0·137	0·311

Some notes on the Scott-Wilson method.

Scott-Wilson [1911] says that the mercuric cyanide reagent remains perfectly clear for months; in my experience it invariably develops a finely divided precipitate soon after preparation. This substance no doubt contains mercury or silver, or both, and must be removed. It is best to make up the reagent at least a week before it is required, and to filter through glass wool before use.

The constituents of the acid mixture are given by Scott-Wilson in "parts," but it is not stated whether these are by weight or by volume. The following mixture has been used in the present investigation: water 550 cc., concentrated sulphuric acid 27 cc., concentrated nitric acid 280 cc.

The precipitation can be carried out in beakers, as the precipitate is removed from these much more easily than from Erlenmeyer flasks. The precipitate after standing adheres very tenaciously to the vessel; it is best removed by rubbing with a glass rod and piece of filter paper; the paper is transferred on to the filter, and is used again in the same way to wipe out the funnel before the titration. If rubber tubing on a glass rod is used, the very fine precipitate gets inside the rubber however closely this be attached.

A Gooch crucible does not seem very suitable for the filtration. If an ordinary glass funnel be used with a porcelain disc, the final removal of the

precipitate is much easier, and the white particles are more visible on glass than on porcelain. The filter paper on the disc is covered with a suspension of talc in water, the talc being well sucked down before beginning the filtration. Such filters will stand the full force of the pump, in spite of the destructive action of the reagent upon filter paper. The precipitate and beaker should be washed five times after wiping the precipitate out of the beaker.

After washing, the material is lifted into an Erlenmeyer flask with a glass rod, and the funnel wiped with filter paper. Not more than 25 cc. of acid mixture should be used in washing down, as much dilution renders the end-point very vague. About 2 cc. of N/5 permanganate are added, and the mixture boiled till it is colourless; the sides of the flask are then washed down with a jet of acid mixture, about 1 cc. of permanganate is added, and the boiling repeated. No concordant results were obtained till this method of decomposing the precipitate was adopted.

The titration is carried out with N/100 KCNS, which can be standardised against silver nitrate. The first faint brown colour is the end-point; three more drops of sulphocyanide produce an obvious reddish-brown tint, and it is well always to obtain this second colour in order to be sure of the first.

Summary.

The oxidation of β-hydroxybutyric acid with sulphuric acid and potassium bichromate in Shaffer's method of estimation gives from 90 to 96 % of the theoretical amount of acetone.

A combination of Shaffer's method with Scott-Wilson's method for the estimation of acetone is described.

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XXXI. COUNTER DIFFUSION IN AQUEOUS SOLUTION.

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(*Received May 4th, 1914.*)

Although we cannot put forward a satisfactory theory for the results of our experiments yet we think that they are of sufficient interest to merit publication—they may at least direct attention to a peculiar feature of diffusion so far undescribed. The following experiments are based upon the discovery of one of us (W. A. O.) that if a strong concentration of solute is allowed to diffuse into some solvent, and if both solution and solvent contain, in addition, another substance in weak concentration, in this case sodium chloride, there is an increase in concentration of the latter dilute substance in the path of the advancing diffusion. If, for instance, there are two solutions, one vertically placed above the other and in contact over a relatively small surface, and if both contain decinormal sodium chloride and one of them, necessarily the lower, contains ammonium sulphate of three molecular strength, then after some days' diffusion it is found that the sodium chloride concentration in the upper fluid is greater than decinormality whilst that in the lower fluid is correspondingly reduced.

Methods.

Vertical glass cylinders were employed each of 23 millimetres internal diameter and with a tap near the middle which had a bore exactly the same diameter as that of the cylinder. The volume from the bottom of the cylinder to the top of the bore of the tap was estimated and a corresponding volume measured off above the closed tap. In the earlier experiments the volume of fluid employed was 100 cm.^{3} below and 100 cm.^{3} above, but we found later that quicker results could be obtained by taking 30 cm.^{3} instead of 100. The lower diffusion fluid was poured into the cylinder until it

rose above the tap. The tap was closed and the upper part of the cylinder cleaned out with distilled water and dried. The upper diffusion fluid was then placed in the cylinder above the tap and a glass stopper inserted. The cylinder, still kept vertical, was then set up in a sheltered part of the laboratory, the tap was turned on, and diffusion allowed to take place at room temperature. When a sufficient interval of time had elapsed the tap was turned, the upper fluid well mixed and poured out. The upper part of the cylinder was then washed with distilled water and dried. The tap was then opened and the lower portion of fluid poured into a separate beaker and well mixed. Sodium chloride, or rather the chloride present, was estimated by the Volhardt procedure. Sulphate was determined gravimetrically as barium sulphate in the usual way and the concentration of ammonium sulphate calculated from the results. Glucose was estimated polarimetrically care being taken as regards mutarotation. The hypobromite method was considered sufficiently accurate for urea. By percentages are meant grams of solute in 100 cm.^{3} of solution.

The following experiments are typical of many others which we performed showing results of strong ammonium sulphate solution in affecting the increase of concentration of chloride.

Experiment I.

Before diffusion—
Above tap, 30 cm.^{3} NaCl 0·58 %.
Below tap, 30 cm.^{3} NaCl 0·57 %, $(NH_4)_2SO_4$ 39·6 %.
After diffusion for seven days—
Above tap, 30 cm.^{3} NaCl 0·63 %, $(NH_4)_2SO_4$ 5·6 %.
Below tap, 30 cm.^{3} NaCl 0·53 %, $(NH_4)_2SO_4$ (by difference) 34·0 %.

Experiment II.

Before diffusion—
Above tap, 30 cm.^{3} NaCl 0·58 %.
Below tap, 30 cm.^{3} NaCl 0·57 %, $(NH_4)_2SO_4$ 39·6 %.
After diffusion for 21 days—
Above tap, 30 cm.^{3} NaCl 0·66 %, $(NH_4)_2SO_4$ 6·0 %.
Below tap, 30 cm.^{3} NaCl 0·46 %, $(NH_4)_2SO_4$ (by difference) 33·6 %.

The differences in the chloride concentration are certainly striking and well outside the limits of error. We next tried the effect of two ammonium sulphate solutions of different concentrations in separate cylinders placed together under similar conditions of temperature and for the same time.

Experiment III.

A. Before diffusion—
Above tap, 30 cm.³ NaCl 0·56 %.
Below tap, 30 cm.³ NaCl 0·56 %, $(NH_4)_2SO_4$ 26·4 %.
After diffusion for 7 days—
Above tap, 30 cm.³ NaCl 0·62 %, $(NH_4)_2SO_4$ 4·1 %.
Below tap, 30 cm.³ NaCl 0·49 %, $(NH_4)_2SO_4$ (by difference) 22·3 %.

B. Before diffusion—
Above tap, 30 cm.³ NaCl 0·56 %.
Below tap, 30 cm.³ NaCl 0·56 %, $(NH_4)_2SO_4$ 52·8 %.
After diffusion for one week.
Above tap, 30 cm.³ NaCl 0·64 %, $(NH_4)_2SO_4$ 7·3 %.
Below tap, 30 cm.³ NaCl 0·46 %, $(NH_4)_2SO_4$ (by difference) 45·5 %.

The stronger solution of ammonium sulphate effected therefore a greater heaping up of the chloride. In order to avoid the difficulties associated with an ionisable salt we tried diffusion with cane sugar and also with glucose.

Experiment IV.

Before diffusion—
Above tap, 100 cm.³ NaCl 0·58 %.
Below tap, 100 cm.³ NaCl 0·58 %, Cane sugar 34·2 %.
After diffusion for seven days—
Above tap, 100 cm.³ 0·60 % NaCl.
Below tap, 100 cm.³ 0·56 % NaCl.

Experiment V.

Before diffusion—
Above tap, 30 cm.³ NaCl 0·58%.
Below tap, 30 cm.³ NaCl 0·57 %, Glucose 36 %.
After diffusion for seven days—
Above tap, 30 cm.³ NaCl 0·60 %, Glucose 5·6 %.
Below tap, 30 cm.³ NaCl 0·54 %, Glucose (by difference) 30·4 % .

Organic non-electrolytes have therefore the same property. On the assumption of a simple kinetic explanation based upon molecular volumes we tried equimolecular mixtures of glucose, $C_6H_{12}O_6$, and urea, CON_2H_4.

Experiment VI.

Before diffusion—
Above tap, 100 cm.³ NaCl 0·56 %, Urea 12 %.
Below tap, 100 cm.³ NaCl 0·54 %, Glucose 36 %.
After diffusion for seven days—
Above tap, 100 cm.³ NaCl 0·56 %.
Below tap, 100 cm.³ NaCl 0·54 %.

Experiment VII.

Before diffusion—
Above tap, 100 cm.³ NaCl 0·57 %, Urea 12 %.
Below tap, 100 cm.³ NaCl 0·58 %, Glucose 36 %.
After diffusion for 14 days—
Above tap, 100 cm.³ NaCl 0·58 %.
Below tap, 100 cm.³ NaCl 0·56 %.

Experiment VIII.

Before diffusion—
Above tap, 100 cm.³ NaCl 1·16 %, Urea 12 %.
Below tap, 100 cm.³ NaCl 1·16 %, Glucose 37·2 %.
After diffusion for 14 days—
Above tap, 100 cm.³ NaCl 1·17 %, Glucose 2·8 %.
Below tap, 100 cm.³ NaCl 1·15 %, Glucose (by difference) 13·4 %.

Experiment IX.

Before diffusion—
Above tap, 30 cm.³ NaCl 0·58 %, Urea 12 %.
Below tap, 30 cm.³ NaCl 0·55 %, Glucose 35 %.
After diffusion for seven days—
Above tap, 30 cm.³ NaCl 0·59 %, Urea (by difference) 10·9 %, Glucose 3·3 %.
Below tap, 30 cm.³ NaCl 0·55 %, Urea 1·1 %, Glucose (by difference) 31·7 %.

The results of this last group are not quite so definite. We tried the same solution as in Experiment IX allowing diffusion to continue for seven weeks but got no greater effect. It will be seen, however, that the differences obtained, though they may extend but little beyond the limits of error, all point in the same direction. We leave the investigation at this stage trusting that some light may be thrown on the subject from the theoretical side.

XXXII. THE CURATIVE ACTION OF AUTOLYSED YEAST AGAINST AVIAN POLYNEURITIS.

By EVELYN ASHLEY COOPER, *Beit Memorial Research Fellow, Lister Institute.*

(*Received May 5th, 1914.*)

In a previous communication [Cooper, 1913] a method was described for the preparation from voluntary muscle of a substance, small amounts of which readily cured polyneuritis in pigeons. The method employed however was not only expensive, but the yield of active substance was very small, so that an attempt has since been made to discover a less costly process of preparing a non-toxic strongly curative substance suitable for use in the treatment of human beri-beri.

Yeast has for some time been known to be very effective in curing polyneuritis in birds, its curative action having been first observed by Schaumann [1910], and Cooper and Funk [1911] subsequently showed that the yeast still retained its curative properties after hydrolysis with 20 % sulphuric acid for 24 hours.

As yeast contains a powerful proteolytic ferment and is easily obtainable in the East, it was thought that possibly a suitable curative solution could be prepared from it by autolysis.

325 gms. of pressed brewers' yeast was accordingly placed in a flask in the hot room (35° C.) for 36 hours, and the yeast was rapidly converted into a brown fluid. This was filtered, and the residual cell-debris washed with a little water. The total volume of the combined filtrate was 270 cc.; 3 cc. of the liquid readily cured pigeons (300 gms.) affected with polyneuritis in 3 to 12 hours, and even 1—2 cc. improved the condition of the birds. As from 3 to 6 gms. of pressed yeast was necessary to bring about complete recovery, it is seen that the solution resulting from autolysis was at least as strongly curative as the original yeast.

A solution of similar potency could be obtained by the addition of 95 per cent. alcohol to the autolysed yeast, but, apart from the fact that the alcohol coagulated the cell-debris and so facilitated filtering, this method had no advantage over the one described above.

By treating the alcohol-free filtrate with basic lead acetate, a copious precipitate was obtained, and the active substance passed almost entirely into the filtrate. In this way much of the colouring matter could be removed, and the highly curative filtrate, although not superior to the original solution therapeutically, can be so readily obtained in large amount that it should be suitable raw material for fractionation with a view to the isolation of the anti-neuritic substance.

Five kilos. of pressed yeast were next autolysed as before for 36 hours, and the filtered solution measured 4500 cc. Three cc. of this readily cured pigeons affected with polyneuritis in a few hours, and the solution stored in the cold room retained its curative power for at least 8 weeks. Birds were then given orally large volumes of this active solution to ascertain if it exerted a toxic action, and the following results were obtained:—

Bird	Weight	Dose	Effect
Pigeon	300 gms.	30 cc. (15 cc. twice in 8 hrs.)	Still healthy 1 week afterwards.
,,	300 ,,	30 ,, ,, ,,	
Chicken	1800 ,,	100 cc. (50 cc. ,, ,,	
,,	500 ,,	36 cc. (18 cc. ,, ,,	

The results show that as much as 10 times the curing dose (3 cc.) given to pigeons had no injurious effect, and even 100 cc. given to the chickens did no harm.

Some experiments were also carried out to ascertain whether a strongly curative solution could be as easily prepared when the yeast was first air-dried at 20° C. To 75 gms. of the dry preparation, which had been stored for six weeks in a dry tin, 250 cc. of water was added, and the yeast allowed to autolyse at 35° C. for 48 hours. The mixture was then filtered, and the insoluble matter washed with water, the combined filtrates measuring 250 cc. Three cc. of the solution again rapidly cured neuritic pigeons. As 1–2 gms. of the dried yeast were required to effect complete recovery, it is seen that the solution was quite as strongly curative as the material from which it was derived. 0·5 cc. of the solution injected subcutaneously rapidly cured a pigeon, and 1–2 cc. had no toxic action.

Air-dried yeast retained its marked anti-neuritic properties even after storage in a dry tin for two years, and after four months' storage still readily autolysed when mixed with water and kept at 35° C.

By autolysing yeast it is thus possible to prepare a highly active solution which being non-toxic and inexpensive should be suitable for the treatment of human beri-beri on a large scale. Owing to the stability of the anti-neuritic substance and proteolytic ferment contained in yeast, as shown above, the yeast can be air-dried, stored under dry conditions, and autolysed as required.

Summary.

1. By the autolysis of brewers' yeast a solution can be obtained which possesses as marked curative properties towards avian polyneuritis as the original yeast.

2. The solution retains its curative power for at least eight weeks and when given orally to birds in doses 10 times as great as the minimum curing dose has no toxic action.

3. Air-dried yeast retains its curative power after storage for two years in a dry tin, and still autolyses after storage for four months.

Conclusion.

The autolysis of brewers' yeast should afford a simple inexpensive method of preparing a non-toxic solution suitable for the oral treatment of human beri-beri.

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XXXIII. A NOTE ON THE BASES OF GASWORKS COAL-TAR WHICH ARE BELIEVED TO BE THE PREDISPOSING CAUSE OF PITCH CANCER, WITH SPECIAL REFERENCE TO THEIR ACTION ON LYMPHOCYTES, TOGETHER WITH A METHOD FOR THEIR INACTIVATION.

PART I. AUXETIC ACTION.

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(*Received May 12th, 1914.*)

It has long been known that gasworks coal-tar, the pitch derived from it and soot (which contains tar), cause chronic dermatitis, warts and ulcers leading to epithelioma, in the skins of the workmen employed at the briquette (patent fuel) works and tar distilleries. This specific predisposition to cancer brought about by these commodities has recently formed the subject of Home Office enquiries. The clinical evidence shows that it is only what is commonly known as coal-tar and the pitch derived from it by distillation that have this action; tar and pitch obtained from the blast-furnaces are harmless. The mechanical irritation caused by both varieties is identical, so that it is evident that some chemical factor is responsible for the predisposition to cancer above referred to, a fact borne out by the incidence of pitch cancer and warts caused by liquid petroleum [Legge, 1911; Lush, 1911; Ross, H. C., 1913, 1].

Although it is well known that gasworks tar differs widely in its chemical composition from blast-furnace tar, the latter being manufactured at a much lower temperature, it must also be remembered that the tar made at the gasworks and coke-ovens which is dangerous, is derived mostly from bituminous coal; the harmless blast-furnace tar, on the other hand, being made from the harder Scotch coal. [Ross, 1913, 2.]

H. C. Ross and J. W. Cropper [1910] put forward a working hypothesis connecting the onset of carcinoma and the predisposition to it with chemical substances called auxetics and kinetics. Auxetics are substances (most of them are amino-bodies) which induce cell-division in white blood cells, such as lymphocytes and other cells; kinetics are another group of substances (including most of the alkaloids) which excite amoeboid movements in cells.

The method of testing for both auxetics and kinetics is to mix the suspected solution with agar jelly, and to spread some blood cells on the surface of films prepared from this jelly. If auxetics are present in sufficient strength the lymphocytes after 10 minutes incubation at 37° will be seen to exhibit characteristic division figures; if kinetics are present, the cells (being examined without incubation) show exaggerated amoeboid movements. It is this test that has been employed in the following chemical experiments; the full details of the method have been described by Ross and Cropper [1911, 1]. In every case the test was repeated several times and throughout the experiments gave consistent results.

The present paper is concerned only with auxetic action, and the clinical bearing of this is not discussed; the question of the isolation of kinetics from tar will be described at a later date. In investigating the question of pitch cancer, Ross and Cropper [1911, 2] have detected auxetics and kinetics in gasworks tar and pitch, in soot and in liquid petroleum they were however unable to detect any kinetic whatever, and only a trace of auxetic in the harmless blast-furnace tar and pitch. A long series of experiments was made by these investigators and the subject is also dealt with in the Report of the second Home Office enquiry on pitch cancer [Lush, 1913].

From observations made by Ross and Cropper it was found that on fractionally distilling tar which had been found capable of yielding an aqueous extract active in inducing cell-division, the fraction distilling between 260°–320° comprised the whole of the active portion, that is to say the activity was associated with the anthracene fraction. Various pure substances known to occur in this fraction were tested by the above observers but found to be without action on lymphocytes. The following experiments were therefore undertaken to see whether the active agent or agents could be isolated.

Experimental.

Isolation of active basic fractions.

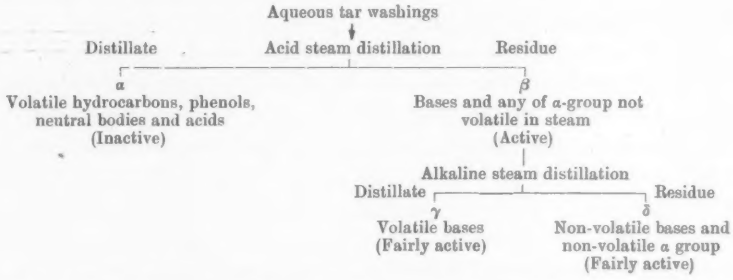
The tar used in the following experiments was a sample of the common variety which causes disease at the tar distilleries and briquette works and was kindly placed at our disposal by Mr Atkinson Butterfield.

Aqueous extracts were faintly acid in reaction.

Preliminary tests were made to ascertain the chemical nature of the substance or substances responsible for this auxetic action and in the first place an aqueous extract of the tar was used.

This was prepared by taking two volumes of water at 60° to 1 volume of tar, shaking for 1 hour, allowing to stand overnight and then centrifugalising or decanting off the aqueous layer. The washings prepared in this way were always faintly acid in reaction.

It was then found that on steam distilling with this original acid reaction, the substances capable of action on lymphocytes remained in the residue, but the kinetic action had apparently been destroyed or inhibited in some way as it was neither in the distillate nor residue. On steam distillation with a faintly alkaline reaction auxetic could be detected in both distillate and residue, that is to say the auxetic is slightly volatile in steam. The two steam distillations were then carried out consecutively on the same sample of washings and the result shown diagrammatically below, pointed to the active constituent being basic in character.



The active substance being therefore apparently of basic nature, acid extracts of the tar were made and it was found that this treatment yielded solutions which had a much stronger action on lymphocytes than had the aqueous ones. Acid extracts were accordingly used in all the following experiments, the acid employed consisting of two volumes of 5 per cent. hydrochloric acid to 1 of tar, and the extraction was carried out at 60° as in the previous case with water.

An attempt was then made actually to isolate the bases from the portion γ in the scheme above by means of chloroplatinic acid in the usual way, but after decomposing the platinum salt by means of sulphuretted hydrogen and then removing the excess of the latter by a current of air, no auxetic action could be detected. One explanation of this may be the adsorption of the active substance by the platinum sulphide which at first was formed in colloidal solution. A further explanation of this loss of activity was afterwards discovered and will be referred to later. The filtrate from the platinum salt was also found to be inactive after the removal of the platinum as above.

A further attempt was made using basic lead acetate as precipitant, decomposing the lead salt with sulphuric acid and then removing excess of acid by means of baryta. Here again no trace of auxetic action remained.

It then seemed necessary to obtain a larger and more concentrated supply of the active substance and methods with this end in view were next tried. It was found that on the addition of 10 per cent. caustic soda to an aqueous extract a precipitate was obtained which redissolved on continued addition of alkali and that this precipitate when filtered off and dissolved up in a little dilute acid (HCl) was a strong auxetic but did not keep well, rapidly losing its activity. In the same way the aqueous and acid extracts of tar likewise lose their activity on long standing in the air. With this method it was extremely troublesome to remove the excess of caustic soda, and if this is not done properly it is impossible to carry out the auxetic test with any hope of success as the alkali kills the cells, and an excess of salts even if neutral must be avoided[1].

The following method was then tried and found to be quite reliable. The tar was extracted as before with twice its volume of 5 per cent. hydrochloric acid, being shaken for one hour, and allowed to stand overnight and then centrifugalised. The supernatant liquor was precipitated with strong ammonia, care being taken not to add excess. A precipitate was obtained which on testing was found to be highly active while the supernatant liquor was inactive. The precipitate was collected, dried in a desiccator and again tested when dry and was still found to be highly active.

It is interesting to note that samples of alkaline condenser liquors and hydraulic main liquors from gasworks, which were kindly given by Dr Colman, were found after removal of sulphur to give the auxetic test. It is not certain whether these materials would be good sources of auxetics, as the latter deteriorate rapidly when exposed to the air in alkaline solution.

[1] The solutions must always be neutralised before testing on lymphocytes. Salts delay the diffusion of substances into cells so that they must not be in excess.

The precipitate from the ammonia may also be taken up in benzene and dried over anhydrous sodium sulphate, but before testing, the benzene must be very thoroughly blown off under a fan or distilled off under reduced pressure; the previous treatment is however to be preferred. In this way 85 g. of bases were extracted from about 1 cwt. of tar. These were distilled under reduced pressure and separated into five fractions.

Fraction	B.-P. at 16 mm.	Weight of distillate
I	0—100°	3·4 g.
II	100—150°	19·2
III	150—200°	18·2
IV	200—220°	8·5
V (Residue)	—	33

Fractions II and III were found to be highly active, I was extremely poor and IV and V were quite inactive.

II and III were then refractionated, but no great difference in activity could be detected in the fractions thus obtained. Both II and III oxidised rapidly in the air and in so doing lost their auxetic power, and this fact presumably accounts for the loss of action previously referred to in the weak aqueous and acid extracts of tar.

Further treatment of basic fractions.

Experiments were next made on the isolation of these active constituents and qualitative experiments showed the presence of nitrogen and possibly of sulphur.

An estimation kindly made by Dr Hartley of this Institute with van Slyke's apparatus showed that the nitrogen was not contained in any side chain but was bound up in the ring.

In this connection it was found that Kahlbaum's pseudocumidine, which is closely allied to the cumidine occurring in coal tar, was very feebly active. The test in this case requires great care as the conditions must be exactly right for any activity to be shown at all.

ψ-cumidine is easily benzoylated in the usual way yielding a white crystalline compound, Mp. 174–5° which has no longer any detectable action on lymphocytes. Attempts were then made to isolate individual bases from the mixture obtained by precipitation with ammonia, by means of benzoyl chloride, but little evidence of benzoylation could be obtained and no crystalline benzoyl compounds could be isolated. Had this been successful it would have been possible to separate the aniline bases from the pyridine bases and to ascertain whether auxetic action was an inherent property of either or both.

The next method tried was fractional crystallisation of the picrates. A saturated solution of picric acid was made, preferably in alcohol, and the basic fraction added drop by drop. A yellow solid at once separated out which crystallised readily from acetic acid. On fractional crystallisation two main fractions were obtained. The first more insoluble portion A, having a Mp. 199°–201°, the second B (after several recrystallisations) Mp. 161°–2°.

Analysis of A gave the following figures.

0·0895 g.; 0·1709 g. CO_2; 0·0219 g. H_2O.
C = 52·11 %, H = 2·71 %.
0·0999 g.; 0·1924 g. CO_2; 0·0252 g. H_2O.
C = 52·52 %, H = 2·80 %.
0·1188 g.; 16·8 cc. N_2 at 16° and 761·75 mm.
N = 16·52 %.
0·1319 g.; 19·1 cc. N_2 at 16° and 760·5 mm.
N = 16·80 %.

The figures obtained for picrate A correspond with the following empirical formula, $C_{22}H_{14}O_9N_6$ which requires

$$C = 52{\cdot}17\ \%\ H = 2{\cdot}76\ \%\ N = 16{\cdot}60\ \%,$$

but nothing further is at present known with regard to the constitutional formula.

The quantity of picrate B, Mp. 161°–2°, obtained was insufficient for analysis.

Experiments on the hydrolysis of picrate A were then undertaken with the object of isolating again and if possible identifying the active base from it, but the results so far have been somewhat unsatisfactory. In the first place the picrate was only hydrolysed with difficulty, and secondly it was found almost impossible to separate the very small amount of free base produced from the metallic picrates formed in the hydrolysis. Only when this was carried out with litharge was any free base obtained and the amount of this was much too small for identification to be possible.

The solution of this free base did however produce cell-division showing that it was one of the active constituents.

The hydrolyses were all carried out in air and possibly better results might be obtained by using an atmosphere of nitrogen, the active bases being very readily oxidised to inactive compounds.

Experiments on the inactivation of the auxetics of coal-tar.

In view of the theory that the susceptibility of pitch workers is due to the presence in the pitch used of the auxetics above described it seemed highly desirable to devise a method whereby these substances might be removed from the tar and pitch or at any rate inactivated. As has been

described above the auxetics can be almost completely removed from the tar by thorough washing with water or dilute acid. These methods however leave the tar in a state which renders its further distillation extremely troublesome and hence have practical objections.

A method has been recently patented by H. W. Robinson [1913] for the treatment of the tar or pitch by formaldehyde whereby the auxetics are inactivated, and an important test on a practical scale is now being made at the briquette works at Cardiff to see if this inactivation of auxetics prevents the disease occurring among the workmen.

The experiments described above, however, have suggested a simpler method of inactivation. It was noticed that aqueous extracts of tar rapidly lost their auxetic power if left exposed to the air for any considerable time. This was especially noticeable if the extracts had been made alkaline and hence the free bases unless dried or kept in an evacuated desiccator rapidly become inactive. The above facts suggested that the loss of activity was due simply to the oxidation of the auxetics and hence experiments were at once commenced with a view to rendering the tar innocuous by means of oxidation methods.

The following experiments illustrate the method finally adopted. 100 g. of tar were heated in a flask in an oil bath to 160° and a blast of air blown through. The tar was then extracted as before with acid, and the extract after neutralisation tested for auxetic action and found to be inactive in every case. A similar experiment was carried out with ozonised air and again the extract of the residue was inactive. The oxidation was quicker in this case than in the experiment where ordinary air was used. The auxetics present can therefore readily be rendered harmless by oxidation according to the method described and it is hoped shortly to try the process on a large scale. The experiments just described refer only to the auxetics, the inactivation of the kinetics being at present under investigation.

Summary.

1. Methods are discussed for the separation of basic constituents of gas-works coal-tar which excite cell division.

2. The bases capable of exerting this action are found to occur in the anthracene fraction of the tar.

3. Two of these bases have been isolated as picrates but have not as yet been satisfactorily identified.

4. A method for the inactivation of these bases in tar is described.

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XXXIV. THE VISCOSITY OF PROTEIN SOLUTIONS. II. PSEUDOGLOBULIN AND EUGLOBULIN (HORSE).

By HARRIETTE CHICK.

From the Lister Institute.

(*Received May 18th, 1914.*)

The following experiments form a continuation of those published recently by Lubrzynska and the author [1914], on the influence of concentration of protein upon the viscosity of horse serum and solutions of crystallised egg and serum albumin (horse). In the present case the investigation has been extended to the pseudoglobulin and euglobulin of horse serum. The significance of both sets of results is discussed at the end of the present paper.

Preparation of the Material.

By pseudoglobulin is signified the protein, soluble in distilled water at its iso-electric point, which is separated from serum by one-half saturation with ammonium sulphate[1] (addition of an equal volume of saturated ammonium sulphate solution to the diluted serum). The term euglobulin is used to indicate the protein insoluble in distilled water at its iso-electric point and therefore precipitated by dilution of the serum and acidification until the reaction is adjusted to the iso-electric point of the protein.

The protein precipitated by one-third saturation with ammonium sulphate (addition of one volume of saturated ammonium sulphate to two volumes diluted serum) is frequently considered to be euglobulin, and this method is a standard one for separating this protein from pseudoglobulin. The precipitate obtained, however, is a mixture of the two, and the total amount is considerably more than is obtained by dilution and acidification. Further, it does not include all the euglobulin, for the precipitate of pseudoglobulin, obtained when the concentration of ammonium sulphate in the filtrate is

[1] There is no reason to suppose that this method sharply separates pseudoglobulin from albumin [see Chick and Martin, 1913].

raised to one-half saturation, is found to contain more protein insoluble in water (euglobulin), than if the latter had previously been removed by dilution and acidification.

Euglobulin. In the present case the euglobulin was always prepared by the last named method. Serum was diluted ten times and the reaction adjusted to the iso-electric point of the euglobulin by means of the addition of a small amount of acetic acid, about 2–3 cc. normal acetic acid being, as a rule, found necessary for each litre of diluted serum. The optimum amount was determined by making preliminary trials with small samples, and was easily recognised by the ease and rapidity with which the precipitate settled. After one to two days' standing the precipitate was separated by centrifuging the deposit, after decanting the top liquor. It was purified three or four times by redissolving (dispersing) in a minimum of caustic soda and precipitating with an exact equivalent of hydrochloric acid. The stronger acid is here employed in place of acetic, because a smaller quantity is found to be necessary and a lower concentration of salt results on neutralising. The final precipitate was washed once with slightly acidified water. For this purpose water, saturated with carbon dioxide and diluted about 30 times with ordinary distilled water, has been found to yield the convenient, slight, acidity.

Pseudoglobulin. The pseudoglobulin, used for the experiment given in Table V, was prepared from diluted horse serum by one-half saturation with ammonium sulphate, after removal of the precipitate given by one-third saturation. The crude precipitate was dissolved in water and twice re-precipitated, the final material being dialysed for two days against running water (tap) and for sixteen days against distilled water, changed daily, in presence of toluene.

The method of precipitation by one-third saturated ammonium sulphate, as stated above, does not remove all the euglobulin. The small proportion remaining can be detected even before the dialysis is commenced, by diluting the material and acidifying with minute amounts of dilute acetic acid, a method by which euglobulin can readily be detected in presence of a small concentration of salt [see Chick, 1913]. After dialysis for nine days, as much as one-tenth of the total protein was found to be insoluble in distilled water. At the completion of the process the material contained about 14 % protein, of which about one-eighth was found to be in this condition. This insoluble material, the presence of which must, I think, be attributed to a gradual degradation of the pseudoglobulin to a water-insoluble condition, shows very remarkable analogies with euglobulin and has been made the

subject of a separate investigation, the result of which will be published later. When the dialysis was complete[1], the insoluble material could be completely separated by diluting and centrifuging. The top liquor was found to be free from insoluble protein and was concentrated over sulphuric acid in a vacuum in order to obtain material of the right strength for the viscosity measurements.

In order to investigate material which had suffered no degradation, dialysis was dispensed with in preparing that used for the experiments in Table VI. The euglobulin was removed in the ordinary way from the serum by dilution (1 in 10) and acidification and a further precipitate separated by addition of ammonium sulphate to one-third saturation (19·8 g. $(NH_4)_2SO_4$ to 100 cc.); ammonium sulphate was then added to the filtrate until one-half saturation was reached (11·3 g. $(NH_4)_2SO_4$ to 100 cc., final density 1·138). The pseudoglobulin was purified as follows. The precipitate was pressed between filter paper to free it from mother liquor, redissolved in water and an equal volume of saturated ammonium sulphate was added. The resulting mixture contained an excess of ammonium sulphate, over one-half saturation, owing to the salt contained in the precipitate. This extra amount was ascertained by boiling a small sample of the mixture, filtering the protein and determining the density of the filtrate. The requisite amount of water could be then calculated and added to the mixture. The precipitate was filtered, freed from mother liquor as far as possible by pressing between filter paper and finally dissolved in a little water. The solution was found to have the following composition: protein 13·75 %, ammonium sulphate 10·46 %. It contained only the faintest trace of insoluble protein (tested by dilution and acidification).

Euglobulin.

Influence of concentration of protein.

The experiment, of which the results are detailed in Table I, was so arranged that the conditions of solution (dispersion) should approximate to those obtaining in normal serum. The euglobulin was dispersed with a small amount of alkali, the concentration of hydrogen ions, 10^{-8} N, approximated to that of serum, and in addition a small amount of sodium chloride was present, about 1 gram per 6 grams protein. The results are shown graphically

[1] During the dialysis of proteins, the reaction gradually approximates to the slight acidity characteristic of the iso-electric point, and as a consequence any euglobulin or "denaturated" protein is gradually precipitated.

in curve *c*, Figure 1, where the results of similar experiments with pseudo-globulin are also plotted, together with those for serum albumin and whole serum [see Chick and Lubrzynska, 1914, p. 61] for purposes of comparison. The much greater viscosity of euglobulin is at once apparent. A solution containing 6·6 % protein has a viscosity of 3·49, i.e. more than twice that shown by solutions of serum albumin or by whole serum of equal protein-content. With higher concentration of protein (12·95 %), the viscosity of euglobulin reaches the high figure of 21·7, a value not approached by the strongest solutions obtained of serum albumin (20·6 % protein, coefficient of viscosity 7·54) or of the proteins of whole serum (18·1 % protein, coefficient of viscosity 6·38).

TABLE I.

Influence of concentration of the protein upon the viscosity of solutions (dispersions) of euglobulin (horse) in dilute sodium chloride solution and alkali.

* Concentration of sodium chloride 2·2 % to 0·5 %.
* ,, ,, hydrogen ions $10^{-8\cdot0}$ to $10^{-8\cdot2}$ normal.
Temperature 25°. Time of flow in viscosimeter for water = 18·5 seconds.

Concentration of protein, %	Mean time of flow in viscosimeter, secs.	Density of solution (H_2O at 25° = 1)	Coefficient of viscosity, $H_2O = 1$
12·95	379·3	1·0582	21·69
10·81	191·7	1·0479†	10·86
9·84	144·9	1·0435	8·17
6·60	62·7	1·0290	3·49
3·27	31·2	1·0141	1·71

* Concentration of salt and alkali was adjusted to give results comparable with those obtained for whole horse serum [see Chick and Lubrzynska, 1914, Table VI, p. 67].
† Interpolated value.

Influence of hydrogen ion concentration and of salt content.

Hardy [1905] found that the viscosity of euglobulin, when dissolved by salt, was much lower than when dispersed by alkali. When small amounts of alkali are used, this is undoubtedly the case, the opalescent material obtained being much more viscous than the clear solution formed when salt is used to dissolve the protein. If, however, the concentration of alkali is increased, the viscosity falls rapidly. This is seen in Table II, where, in Experiment I, the result is given of three determinations of viscosity in solutions containing 5·6 % protein, but with concentration of hydrogen-ions falling from $10^{-7\cdot46}$ N to $10^{-10\cdot02}$ N. Corresponding to this change in reaction, there is a fall in the coefficient of viscosity from 12·17 to 3·62. The last

result is not greatly in excess of that obtained in Experiments II and III which deal with solutions of salt-globulin of the same protein concentration. The reaction in these two instances is not far removed from that of the iso-electric point which was determined by Michaelis and Rona [1910] to be at a concentration of hydrogen-ions equal to about 10^{-6} N. The direct influence of salt in lowering viscosity[1] is seen by a comparison of Experiment IV with Experiment I. In the former case, a small concentration of salt, 1 %, reduced the coefficient of viscosity from about 12·2 to 2·4 in presence of, roughly, the same concentration of hydrogen-ions. The figures for Experiments II and IV are interpolated values obtained from curves *b* and *c* respectively, in Fig. 1.

TABLE II.

Influence of concentration of hydrogen-ions upon the viscosity of euglobulin (horse) (a) dispersed by alkali, Exp. I; (b) by NaCl, Exps. II and III, and (c) by alkali + NaCl, Exp. IV.

Concentration of protein 5·68 %.
Temperature 25°.

Exp.	Concentration of NaCl, %	Concentration of alkali, NaOH, in terms of normality	Concentration of hydrogen-ions, in terms of normality	Density of the solution (H_2O at 25° = 1)	Coefficient of viscosity (H_2O, or salt solution, = 1)
I	—	—	$10^{-7.46}$	1·0164	12·17
	—	0·01	$10^{-9.34}$	,,	6·84
	—	0·02	$10^{-10.02}$	,,	3·62
II	3·6	—	$10^{-5.7}$	—	2·65*
III	3·5	—	$10^{-6.2}$	—	2·77†
IV	1·0	—	$10^{-8.1}$	—	2·39*

* Interpolated values from curves *b* and *c*, Fig. 1, see also Table I and Table III (Exp. II).
† See Exp. I, Table III.

In Table III are given the results of two experiments showing the influence of protein concentration when the euglobulin, at or about its iso-electric point, is dispersed by means of salt alone. It is seen that, when the concentration of protein is sufficiently high, very high values are obtained for the coefficient of viscosity. For example, in Experiment II, when the protein concentration was 13·2 %, the coefficient of viscosity was 29·56.

From a comparison of Tables II and III with Table I, which deals with an experiment in which the conditions obtaining in serum were closely imitated, it is evident that the euglobulin in serum must be regarded as salt-globulin.

[1] This influence would appear even more marked if, in calculating the concentration of protein in the solutions of salt-globulin, any allowance were made for the water appropriated by the salt.

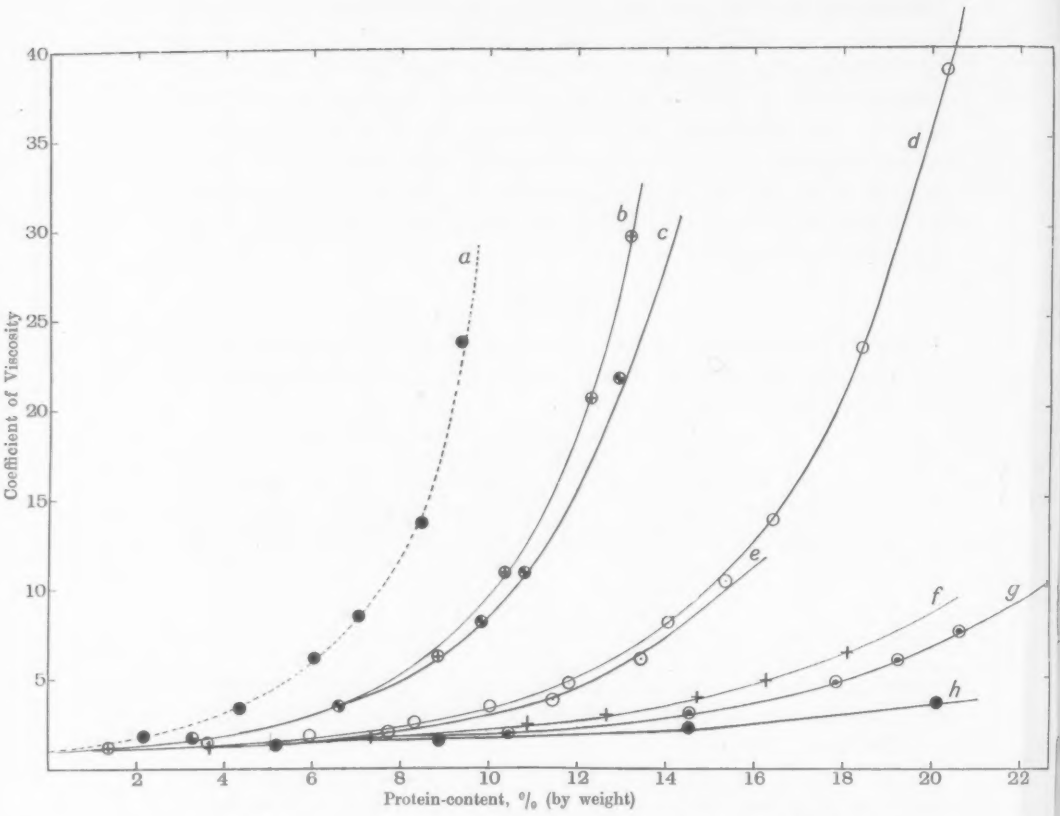

Fig. 1. Influence of protein concentration upon the viscosity of various protein solutions.

Curve *a* ----●---- , sodium caseinogenate [Chick and Martin, 1913].
,, *b* —⊕— , euglobulin (salt). (See Exp. II, Table III.)
,, *c* —◉— , euglobulin (salt + alkali). (See Table I.)
,, *d* —○— , pseudoglobulin. (See Table V.)
,, *e* —⊙— , pseudoglobulin (salt). (See Table VI.)
,, *f* —+— , whole serum (horse). [Chick and Lubrzynska, 1914, Table VI.]
,, *g* —⊙— , serum albumin. [,, ,, ,, ,, ,, IV.]
,, *h* —●— , egg albumin. [,, ,, ,, ,, ,, I.]

TABLE III.

Influence of concentration of protein upon the viscosity of euglobulin (horse) dissolved (dispersed) in sodium chloride solutions.

Exp. I. Concentration of hydrogen-ions $= 10^{-6.2}$ normal.
Exp. II. ,, ,, ,, $= 10^{-5.5}$ to $10^{-6.0}$ normal.
Temperature = 25°.
Mean time of flow for water = 55·4 seconds.

Exp.	Concentration of NaCl, %	Concentration of protein, %	Mean time of flow in viscosimeter, secs.		Density (H_2O at 25° = 1)		Coefficient of viscosity, H_2O containing 3 % NaCl = 1
			Euglobulin solution	Salt solution (3·0 % NaCl)	Euglobulin solution	Salt solution (3·0 % NaCl)	
I	3·0	8·02	241·1	57·6	1·045	1·0211	4·28
	3·7	5·82	142·3	,,	1·038*	,,	2·48
	4·3	3·62	95·2	,,	1·032	,,	1·62
	3·5	1·44	68·6	,,	1·025	,,	1·18
				(3·6 % NaCl)		(3·6 % NaCl)	H_2O containing 3·6 % NaCl = 1
II	3·6	13·20	1654·1	58·1	1·0650	1·0257	29·56
	,,	12·32	1153·3	,,	1·0626*	,,	20·56
	,,	10·57	612·3	,,	1·0574*	,,	10·87
	,,	8·86	352·8	,,	1·0522*	,,	6·23
	,,	6·58	189·4	,,	1·0451	,,	3·32
	,,	1·36	69·1	,,	1·0298*	,,	1·19

* Interpolated values.

Influence of Temperature.

The solution used to investigate the influence of temperature contained 10·81 % protein and 1·82 % sodium chloride, and had a hydrogen-ion concentration equal to 10^{-8} N, the conditions being so arranged as to approximate to those obtaining in normal serum. The viscosity at various temperatures is expressed in relation to that of a 1·82 % solution of sodium chloride, estimations of the latter being made in the same viscosimeter at points over the same range of temperature. The results were expressed in a smoothed curve, from which the values in the 3rd column of Table IV were obtained. With a rise of temperature from 2·1° to 41·3°, the coefficient of viscosity was decreased to less than one quarter (see Table IV), an effect very much greater than that obtained with the other serum proteins investigated. A solution of serum albumin containing 20·65 % protein had its viscosity reduced by only about 30 % for a similar change of temperature, and while, for the whole proteins of serum, the effect of temperature was somewhat greater, it was insignificant compared to the result obtained for euglobulin.

TABLE IV.

Influence of temperature upon the viscosity of solutions (dispersions) of euglobulin (horse) in dilute sodium chloride and alkali.

Concentration of sodium chloride = 1·82 %.
,, ,, hydrogen-ions = $10^{-8.63}$ normal.
,, ,, protein = 10·81 %.
Density of protein solution = 1·0479.
,, ,, 1·82 % NaCl solution = 1·0127.

Temperature, °C.	Mean time of flow in viscosimeter, secs.		Coefficient of viscosity (1·82 % NaCl solution at the same temperature = 1)
	Euglobulin solution	1·82 % solution of NaCl (from curve)	
2·1	939·8	36·3	26·79
9·1	532·4	29·5	18·67
17·4	288·7	24·1	12·39
25·0	198·1	20·8	9·85
32·1	138·6	18·6	7·71
41·3	93·6	16·2	5·97

The great influence of rise of temperature in lowering viscosity is characteristic of emulsoid colloids, perhaps the most striking example being that of solutions of gelatin. The difficulty experienced in filtering serum and other solutions containing proteins is also lessened to a surprising extent on raising the temperature owing to the fall in viscosity. With dilute solutions, of low viscosity, the effect may not be much in excess of that observed for water itself, or for solutions of crystalloids, but it becomes progressively greater with increase in concentration of the colloids and consequent rise in viscosity. For example, a solution of egg-albumin containing 7·04 % protein (coefficient of viscosity = 1·36 at 25°) showed a change of viscosity only about 5 % greater than that of distilled water when the temperature was raised nearly 40 degrees centigrade. When the strength of protein was increased to 20·1 % (coefficient of viscosity = 3·58 at 25·4°) and 28·15 % (coefficient of viscosity at 25·4° = 10·01) the change in the value of the coefficient was about 23 % and 54 % respectively for a similar change in temperature [see Chick and Lubrzynska, 1914, Table II]. In the case of whole serum (protein = 7·68 %; coefficient of viscosity = 1·87 at 25°), the coefficient of viscosity was decreased nearly 14 %, and with concentrated serum (18·1 % protein, coefficient of viscosity = 6·34 at 25°) the fall in value was about 51 % on raising the temperature about 40° [Chick and Lubrzynska, 1914, Table VII].

It is therefore quite consistent that solutions of pure euglobulin, with their very high viscosity, should suffer an even greater change in this respect with alteration of temperature. An interpretation of the phenomenon will be suggested later (see p. 277).

Pseudoglobulin.

Influence of concentration of protein.

The results of viscosity measurements with solutions of pure dialysed pseudoglobulin are set forth in Table V. The method by means of which it was freed from the water-insoluble material, formed during its lengthy preparation, has already been described (p. 263). The results are also graphically shown in curve *d* of Figure 1. It will be seen that, as regards viscosity, solutions of pseudoglobulin occupy a position intermediate between those of euglobulin and of serum albumin. For solutions of equal protein content, the viscosity of those of pseudoglobulin falls far below those of euglobulin and this is especially marked in the stronger solutions. For example, a solution containing 10 % protein, exhibits a viscosity less than one-third of that of a solution of euglobulin of equal strength. At the same time, the viscosity is considerably in excess of that of serum albumin, a 10 % solution being nearly twice and a 20 % solution about five times as viscous [see Chick and Lubrzynska, 1914, p. 65].

TABLE V.

Influence of concentration of the protein upon the viscosity of solutions of pseudoglobulin (horse) dialysed.

Temperature 25°.
Time of flow in viscosimeter for water = 48 secs.
Concentration of hydrogen-ions = $10^{-6.8}$ to $10^{-6.9}$ normal.

Concentration of protein, %	Mean time of flow in viscosimeter, secs.	Density of the solution (H_2O at 25° = 1)	Coefficient of viscosity (H_2O = 1)
20·37	1753·6	1·0619	38·79
18·45	1059·7	1·0558*	23·31
16·43	628·8	1·0490	13·74
14·06	370·8	1·0425	8·05
11·82	218·0	1·0341	4·70
10·04	160 1	1·0289*	3·43
8·32	121·5	1·0239*	2·59
5·95	87·5	1·0169	1·85
3·61	66·4	1·0102	1·40

* Interpolated values.

Influence of ammonium sulphate.

In Table VI are given the results of an experiment with un-dialysed material containing the salt which separated with the protein phase during precipitation by half-saturated ammonium sulphate. The insoluble protein present amounted only to a slight trace. The concentration of ammonium sulphate was equal to 10·4 %, and, in order to compare the results with those given in Table IV, the percentage concentration of protein is reckoned, not as grams per 100 grams of the system but as grams per 100 grams protein and water, see 3rd column Table VI.

TABLE VI.

Influence of concentration of protein upon the viscosity of solutions of pseudoglobulin (horse) containing ammonium sulphate.

Temperature 25°.
Time of flow in viscosimeter for 10·3 % $(NH_4)_2SO_4$ = 53·9 seconds.
Time of flow for water = 48 seconds.
Concentration of hydrogen-ions = $10^{-6.8}$ normal.
Concentration of $(NH_4)_2SO_4$ = 10·4 %.

Concentration of protein		Mean time of flow in viscosimeter, secs.	Density of solution (H_2O at 25° = 1)	Density of H_2O containing 10·3 % $(NH_4)_2SO_4$	Coefficient of viscosity, H_2O containing 10·3 % $(NH_4)_2SO_4$ = 1
G. in 100 g. total system	G. in 100 g. water + protein				
13·75	15·35	535·2	1·1025	1·0601	10·33
12·05	13·45	312·6	1·0972*	,,	6·00
10·26	11·45	198·8	1·0917*	,,	3·80
6·93	7·73	106·8	1·0814	,,	2·02
4·16	4·65	75·3	1·0729*	,,	1·41

* Interpolated values.

As a matter of fact, for accurate comparison, some allowance should also be made for the water appropriated by so large a proportion of salt, but that is impossible to estimate. While the effect of salt in lowering viscosity is much less than that obtaining in the case of euglobulin, its influence is evident from a comparison of Tables V and VI, or of curves *d* and *e*, Figure 1.

SIGNIFICANCE AND INTERPRETATION OF RESULTS.

From a survey of the foregoing results, it is seen that, as regards their viscosity, solutions of the three proteins of serum, albumin, pseudoglobulin and euglobulin, form a series varying from comparatively labile fluids, resembling those of crystalloids, in case of albumin, to liquids of considerable

viscosity, in the case of euglobulin. A concentration of protein of at least 10 % is necessary to reveal the colloidal nature of serum albumin and leads to excessively high viscosities with the other two proteins.

The interpretation of the characteristic phenomena displayed by solutions of these proteins may be found in the two-phase nature of the system. Proteins are "hydrophile emulsoids" and the actual volume of the disperse phase may be assumed to be much greater[1] than that indicated by the solution volume of the protein, as determined from the density of the solution.

Hatschek [1910, 1911] has developed a theory of the viscosity of two-phase systems based upon his own observations of the viscosity of oil-water emulsions. He has shown that when the volume of the oil approaches 70 % of the total volume, at which point the oil particles touch one another, there is an enormous increase in viscosity. This, in his opinion, is due to the inability of the oil particles to roll upon one another under the influence of a shearing force; as a result they suffer deformation. From mechanical considerations he has found that, when the volume of the oil is more than one-half the total volume, the viscosity of such a two-phase system can be expressed in terms of the phase ratio as follows:

$$\eta = \eta' \frac{\sqrt[3]{A}}{\sqrt[3]{A}-1} \quad \ldots\ldots(1),$$

where η = the viscosity of the system,

η' = the viscosity of the continuous phase,

V = volume of the system,

v = volume of the disperse phase,

and $A = \frac{V}{v}$ = the phase ratio.

If the viscosity of the continuous phase be taken equal to 1·0

$$\eta = \frac{\sqrt[3]{A}}{\sqrt[3]{A}-1} \quad \ldots\ldots(2),$$

or

$$A = \left(\frac{\eta}{\eta-1}\right)^3 \quad \ldots\ldots(3).$$

According to this expression, the viscosity of the system is independent of the viscosity of the disperse phase and of the size of its particles, but depends upon the relative volume of the two phases.

The formula was tested by Hatschek [1911, 1913] in the case of oil-water emulsions, of which the composition was accurately known. When

[1] In this connection, it is of interest to note that Findlay and Creighton [1911] found that the solubility of oxygen, at atmospheric pressure, in de-aerated serum was only about one-fifth as great as in water; in the case of nitrogen, the difference was even greater.

the emulsion was sufficiently concentrated, the known volume of oil emulsified was found to be in close agreement with the value calculated by means of the above formula.

Hatschek [1912] has found the above formula also applicable to colloidal solutions of the emulsoid type, in cases where the disperse phase occupies more than one-half the total volume of the system, i.e. where A is less than 2. The two instances selected by him are solutions of glycogen, using the viscosity measurements of Bottazzi and d'Errico [1906], and those of caseinogen, using the results of Chick and Martin [1912]. The value of $A\left(=\frac{V}{v}\right)$ was reckoned by means of the formula (3) given above, V being the volume occupied by 100 grams of the system. The value of $A'\left(=\frac{V}{c}\right)$, where c is the weight of dissolved substance, could be calculated from the measured density of the system and the known concentration of the colloid. The ratio $\frac{A'}{A}\left(=\frac{v}{c}\right)$, expressing the volume occupied by unit weight of the dissolved substance, was then calculated and, in case both of glycogen and caseinogen, Hatschek found a very fair constancy in value, when A was less than 2.

One of the most important results of Hatschek's expression is that, given the viscosity, not only is a method afforded of determining the phase ratio, but from the latter, given the concentration of the colloid and the density of the system, a calculation can, for the first time, be made of (a) the volume occupied, and (b) the amount of water taken up, by unit weight of a hydrophile colloid.

In the case of caseinogen, the value of (a) is equal to 9·3[1], that is to say each gram of caseinogen in colloidal solution occupies a volume equal to 9·3 cc. having taken up 8·6 cc. water (solution volume of caseinogen = 0·70). It follows therefore that even in so comparatively dilute a solution as 6 % (100 grams of the system occupying 98 cc.) the caseinogen phase would occupy 56 cc. or 57 % of the total volume.

Using Hatschek's formula the values of $\frac{v}{c}$ for all the serum proteins used in the present investigation have been calculated and the results for euglobulin and pseudoglobulin are given in Tables VII and VIII and IX respectively. Table X contains a similar set of results for serum albumin, using the viscosity measurements published previously [Chick and Lubrzynska, 1913].

[1] The mean value given by Hatschek, 9·52, is slightly too high, the density of the system being neglected in evaluating A'.

TABLE VII.

Volume occupied by the euglobulin phase in solution at 25° C.

(*a*) Dispersed with salt and alkali (see Table I).
(*b*) Dispersed with alkali (see Table II).

	Concentration of protein, % = c	Concentration of hydrogen-ions, in terms of normality	Concentration of NaCl, %	Density of system = δ	Coeff. of viscosity = η	A = vol. of 100 g. system / vol. disperse phase ($=v$) = $\left(\frac{\eta}{\eta-1}\right)^3$	A' = vol. of 100 g. system / weight of dissolved substance ($=c$) = $\frac{100}{\delta \times c}$	Volume occupied by 1 g. dissolved substance = $\frac{A'}{A} = \frac{v}{c}$	Mean value of $\frac{v}{c}$
(*a*)	12·95	$10^{-8.1}$	2·2	1·0582	21·69	1·152	7·298	6·335	
	10·81	,,	1·8	1·0479	10·86	1·336	8·828	6·608	6·51
	9·84	,,	1·7	1·0435	8·18	1·479	9·738	6·584	
	6·60	,,	1·1	1·0290	3·487	2·757	14·72	5·339	
	3·27	,,	0·5	1·0140	1·710	13·96	30·13	2·158	
(*b*)	5·68	$10^{-7.5}$	—	1·0164	12·17	1·294	17·32	13·39	
	,,	$10^{-9.3}$	—	,,	6·84	1·608	17·32	10·77	
	,,	$10^{-10.0}$	—	,,	3·62	2·638	17·32	6·57	

TABLE VIII.

Volume occupied by the euglobulin phase in salt solution at 25° C.
(See Table III, Exp. II.)

Concentration of protein, % = c	Density of the system = δ	Coefficient of viscosity = η	A = vol. of 100 g. of system / vol. of disperse phase ($=v$) = $\left(\frac{\eta}{\eta-1}\right)^3$	A' = vol. of 100 g. of system / wt. of dissolved substance ($=c$) = $\frac{100}{\delta \times c}$	Volume occupied by 1 g. dissolved substance = $\frac{A'}{A} = \frac{v}{c}$	Mean value of $\frac{v}{c}$
13·20	1·0650	29·56	1·109	7·114	6·415	
12·32	1·0626	20·56	1·162	7·640	6·574	6·51
10·57	1·0574	10·87	1·336	8·948	6·698	
8·86	1·0522	6·23	1·690	10·73	6·346	
6·58	1·0451	3·32	2·930	14·54	4·964	
1·36	1·0298	1·19	245·5	71·40	0·291	

TABLE IX.

Volume occupied by the pseudoglobulin phase in solution at 25° C. (*dialysed*). (*See Table V.*)

Concentration of protein, % $= c$	Density of the system $= \delta$	Coefficient of viscosity $= \eta$	$A =$ Total vol. of system / vol. of disperse phase $(= v) = \left(\frac{\eta}{\eta - 1}\right)^3$	$A' =$ Total vol. of system / wt. of dissolved substance $(= c) = \frac{100}{\delta \times c}$	Volume occupied by 1 g. dissolved substance $= \frac{A'}{A} = \frac{v}{c}$	Mean value of $\frac{v}{c}$
20·37	1·0619	38·79	1·082	4·623	4·272	4·50
18·45	1·0558	23·31	1·141	5·133	4·499	
16·43	1·0490	13·74	1·254	5·803	4·627	
14·06	1·0425	8·05	1·488	6·822	4·585	
11·82	1·0341	4·70	2·050	8·180	3·990	
10·04	1·0289	3·43	2·813	9·682	3·441	
8·32	1·0239	2·59	4·322	11·74	2·716	
5·95	1·0169	1·85	10·31	16·53	1·603	
3·61	1·0102	1·40	42·86	27·42	0·640	

TABLE X.

Volume occupied by the serum albumin phase in solution at 25° C. [*See Chick and Lubrzynska*, 1914, p. 65.]

Concentration of protein, % $= c$	Density of the system $= \delta$	Coefficient of viscosity $= \eta$	$A =$ vol. of 100 g. of system / vol. of disperse phase $(= v) = \left(\frac{\eta}{\eta - 1}\right)^3$	$A' =$ vol. of 100 g. of system / wt. of dissolved substance $(= c) = \frac{100}{\delta \times c}$	Volume occupied by 1 g. dissolved substance $= \frac{A'}{A} = \frac{v}{c}$	Mean value of $\frac{v}{c}$
20·65	1·0593	7·538	1·534	4·572	2·980	2·81
19·24	1·0555	5·875	1·751	4·924	2·838	
17·85	1·0513	4·763	2·028	5·329	2·628	
14·54	1·0413	3·025	3·332	6·605	1·982	
10·45	1·0296	1·952	8·618	9·295	1·078	
5·19	1·0153	1·316	72·20	18·98	0·263	
2·59	1·0075	1·128	684·0	38·32	0·056	

In all cases, with the exception of the experiment set forth in Table VII *b*, which will be discussed separately later on, the value $\frac{v}{c}$ remained constant when the disperse phase occupied more than one-half the total volume. It was greatest in the case of euglobulin, one gram of which, in salt solution, was calculated to have a volume of 6·5 cc. while each gram of dissolved serum albumin was found only to occupy 2·81 cc., pseudoglobulin being intermediate between the two $\left(\frac{v}{c} = 4{\cdot}52\right)$. The solution volume of these

TABLE XI.

Water taken up by various proteins in the formation of colloidal solution at 25° C.

Protein	Density of the protein in solution at 25° (H_2O at 25° = 1)	Solution volume (as calculated from the density of the system)	Volume occupied by 1 g. protein in solution, cc.	Water associated with 1 g. protein, when in solution, cc.
Serum albumin (crystallised)	1·38	0·72	2·81	2·09
Pseudoglobulin	1·39	0·72	4·50	3·78
Euglobulin (salt)	1·42	0·70	6·51	5·81
Sodium caseinogenate	1·43	0·70	9·33	8·63
Egg-albumin (crystallised)	1·36	0·73	2·30	1·57

proteins can be calculated from the density of their solutions and hence the actual amount of water associated with each gram of protein can be calculated. These values vary from 5·8 cc. in the case of euglobulin to 3·8 cc. and 2·1 cc. for pseudoglobulin and serum-albumin respectively. These figures are all collected in Table XI, those for caseinogen and egg-albumin being added for purposes of comparison.

In order to institute useful comparison with the other proteins in serum, solutions of euglobulin in presence of salt have alone been taken into account in the preceding paragraph, because, in normal serum, it is under those conditions that this protein exists. When euglobulin is dispersed by alkali alone, its viscosity shows very remarkable variations, as may be seen from the results of Exp. I in Table II, where the euglobulin was practically salt-free. Under these circumstances the degree of viscosity depends upon the amount of alkali employed, being comparatively high at first and rapidly falling with increasing concentration of alkali and hydroxyl-ions. Increase of the latter from near the neutral point to a concentration of about 10^{-4} N (hydrogen-ion concentration 10^{-10} N) was accompanied, in the case of a 5·68 % euglobulin dispersion, by a fall in the viscosity coefficient from 12·17 to 3·62, which latter number is near the figure obtained for salt-globulin of the same protein-concentration. This decrease in viscosity is accompanied by a visual change, the globulin mixture, which remains in the form of a precipitate at concentrations of hydrogen-ions from about 10^{-5} to 10^{-6} N, changing to a thick, opalescent fluid at the neutral point (conc. $H^+ = 10^{-7}$ N) and to a thin, clear liquid at a concentration of hydrogen-ions equal to about 10^{-10} N. The observed change in size of the protein particles would, according to the theory of Hatschek, be unaccompanied by any change in viscosity unless there were a concomitant change in phase-ratio. In other

words, we must suppose that, in the more alkaline solution, the globulin phase contains less water and that the amount appropriated by the protein steadily increases as the iso-electric point is approached, with a consequent great increase in the volume of the disperse phase. In Table VII *b* the calculated value of $\frac{v}{c}$, the volume occupied by one gram protein in solutions of varying alkalinity, is seen to vary from 13·4 at the neutral point to 6·6 in the most alkaline solution employed. The latter figure, which is practically the same as that obtained for salt-globulin, is probably too low, for, in this case, the volume of the disperse phase, as ascertained by Hatschek's formula, is less than one-half the total volume and the calculation is therefore not strictly permissible.

In the presence of a small concentration of salt, the water appropriated by the globulin phase appears to be nearly independent of the reaction, the calculated value of $\frac{v}{c}$ being the same in the case of the two experiments in Table I and Table III, when the hydrogen-ion concentration was varied from 10^{-6} N to 10^{-6} N (see Tables VII *a* and VIII respectively).

The fact that the addition of alkali to a protein, with the consequent formation of protein salt and increase of protein ions, should be accompanied by a *lowering* of viscosity, and the conception that this is due to a less degree of association of the protein with water is opposed to the results of similar investigations with other proteins. Lacqueur and Sackur [1903] showed that increase in alkalinity led to an *increase* in viscosity in case of sodium caseinogenate. Pauli and Handovsky [1909 and 1910], working with the proteins of ox-serum, have shown that an addition of either acid or alkali leads to a preliminary rise in viscosity, followed by a fall as the concentration of either acid or alkali is further increased. Pauli and Falek [1912] have demonstrated the same phenomenon in case of gelatin solutions, and the work of Spiro [1904], Fischer [1910] and Chiari [1911] has shown that the water imbibed by gelatin increases rapidly as the reaction is made either more acid or more alkaline than the iso-electric point.

The general influence of salts[1] in lowering viscosity of protein solutions (and imbibition of water by gelatin), presumably due to their water-withdrawing capacity, was also demonstrated by the above observers and has been confirmed in the present work. The effect in the case of euglobulin is of great importance and has been fully discussed above; it is also well marked in the case of pseudoglobulin.

[1] What is possibly an analogous phenomenon with alcohol has been demonstrated by Brailsford Robertson [1912]. He found that the viscosity of sodium caseinogenate solutions (about 3 %) was lowered when the proportion of alcohol present was raised from 50 % to 75 %.

Influence of temperature. In the light of the theory developed above, the very marked influence of temperature upon the viscosity of protein solutions (see Table IV above, and Tables II, V and VII, Chick and Lubrzynska [1914]) may be explained by a gradual loss of water from the protein phase as temperature is raised. It is usual, with most substances of crystalloid nature, to find less water needed for the solution of each gram of the solute at a higher temperature, and the same property may also be assumed for substances of a colloidal nature. On such an assumption, the greater the initial appropriation of water, either due to the nature of the colloid or to its high concentration, the greater will be the loss of water from the disperse phase on rise of temperature and the diminution in its volume; in consequence the greater will be the fall in viscosity.

The experimental facts are in accord with this conception. With solutions of egg-albumin, where a comparatively small amount of water is assumed to enter into the composition of the disperse phase, the effect of temperature upon the viscosity coefficient is found to be negligible except in the case of very concentrated solutions (20 % and upwards). On the other hand, comparatively dilute solutions of euglobulin, the most "hydrophile" of the proteins studied, show very marked changes in viscosity with alteration of temperature (see Table IV). In the following manner, I have attempted to estimate the volume occupied by one gram of (salt) euglobulin in solution at various temperatures. From curve *c*, Fig. 1, it is possible to determine the concentration of solutions which, at 25°, would possess the same viscosity coefficients as those determined for the 10·81 % solution at the various temperatures (see the 4th column, Table IV). In the case of these solutions, at 25°, the concentration (in %) multiplied by 6·51, gives the volume (in cc.) occupied by the disperse phase. The same values for the volume of the disperse phase will apply to the 10·81 % solution at the temperatures where the viscosity corresponds, if changes in viscosity due to alteration of temperature are assumed to be the result only of changes in phase-ratio (and if the differences in density of the solutions, caused by differences in temperature and concentration, are disregarded). In this way, the volume of one gram of euglobulin, in salt solution, at 2·1° has been estimated at 8·3 cc. and that at 43° at 5·3 cc.; at 25° the volume has already been calculated as 6·51 cc.

Application of the above results to the salting out of proteins.

The results of the preceding experiments, and the conceptions derived from them, throw a very interesting and illuminating light upon the phenomena displayed in the precipitation of proteins by neutral salts. They explain very clearly the influence of increased protein concentration, both in raising the proportion of protein thrown out by a given concentration of salt, and in lowering the limit of salt concentration at which precipitation occurs.

The values obtained, by the use of Hatschek's formula, for the amount of water presumably associated with unit weight of the various serum proteins in formation of colloidal solutions, yield a satisfactory explanation of the well-known precipitation limits of these proteins. The euglobulin, needing most water for its colloidal solution, is first driven out as water is withdrawn during any salting-out process; a higher concentration of salt is necessary to throw down the pseudoglobulin; while the albumin, which appropriates comparatively little water, is the last to be precipitated.

The current notion that albumin is less readily precipitated than the other proteins of serum, because it has a "greater affinity" for water, is thus seen to be erroneous. It is probably more consistent with the truth to affirm that the albumin, needing the exclusive use of less water for its own solution, is less liable to suffer precipitation when competition occurs.

Summary.

1. The viscosity of solutions of pseudoglobulin and euglobulin from horse serum has been investigated as regards the influence of:

(*a*) Concentration of protein.
(*b*) Temperature.
(*c*) Salt-content.
(*d*) Hydrogen-ion concentration.

The results of similar experiments with serum albumin and whole serum, published previously by Chick and Lubrzynska [1914], are included in the general survey of the results.

2. In all cases, increase in protein concentration is accompanied by a disproportionately great increase in the viscosity of the solution. The effect is greatest in case of euglobulin, solutions of which exhibit a high viscosity at a comparatively low protein content. It is least in case of serum albumin, which, for strengths of protein under about 10 %, behaves almost as a

crystalloid. Pseudoglobulin is intermediate between the other two proteins in this respect.

3. The viscosity of euglobulin solutions is dependent upon the manner in which solution (dispersion) of the protein is obtained. In case of "alkali globulin," where no salt is present, the viscosity of the solution depends upon the degree of alkalinity, falling rapidly with decrease in concentration of hydrogen-ions and distance from the iso-electric point. The viscosity of solutions of "salt-globulin" (NaCl) is considerably lower than that of alkali-globulin of equal protein and hydroxyl-ion content; in this case the viscosity is largely independent of the concentration both of salt (if above a small minimum, 0·5 to 1·0 %) and hydroxyl-ions. Euglobulin in serum is in the condition of salt-globulin.

4. The presence of a salt ($(NH_4)_2SO_4$) lowers the viscosity of pseudoglobulin solutions; it is without influence on the viscosity of solutions of albumin (egg) in concentrations up to 7 %.

5. The viscosity of protein solutions is decreased with rise of temperature frequently to a degree far in excess of that displayed by water or solutions of crystalloids. The greater the viscosity of the solution, the greater is the temperature effect, which is thus much enhanced in solutions of high protein concentration and most marked in case of euglobulin.

6. An interpretation of the above results is found in the two-phase nature of the systems studied. By means of Hatschek's formula, which gives an expression for phase-ratio in terms of viscosity, it is possible to calculate the relative volumes of protein- and water-phase in the more concentrated solutions employed. Hence values can be obtained for the volume occupied by one gram of the various proteins when in solution. These were found to show a satisfactory constancy and to be 2·8 cc., 4·5 cc. and 6·5 cc. for serum albumin, pseudoglobulin and salt-euglobulin respectively, at 25°. (The value obtained for alkali-euglobulin varied from 13·9 cc. to 6·6 cc. according to the alkalinity of the solution.) The solution volume of these proteins, reckoned from the density of their solutions, is equal to 0·7; the amount of water taken up by one gram of the protein at 25° would therefore be 2·1, 3·8 and 5·8 cc. respectively.

7. The conclusions given in no. 6 afford a satisfactory explanation of (*a*) the influence of protein concentration upon both the amount of protein precipitated, and the limit of salt concentration required to commence precipitation, in case of a pure protein; and (*b*) the relative order in which the above three proteins are precipitated from a mixture with increasing concentration of salt.

8. Hatschek's theory explains the observed disproportionate increase in viscosity of protein solutions with increase in concentration of the protein, the volume of the disperse phase being increased at the expense of the continuous phase. The relative magnitude of the phenomenon in case of the three proteins investigated is also interpreted.

9. The influence of temperature upon the viscosity of protein solutions would be explained by assuming a less degree of hydration of the colloid at higher temperature (an analogous phenomenon is encountered with crystalloids). A calculation has been made of the volume occupied by one gram of the protein at various temperatures in case of euglobulin. These volumes vary from 8·3 cc. at 2° to 5·3 cc. at 41°.

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